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High Fat Diet Affects The Dopamine Reward System: Importance Of Sex And Critical Developmental Periods

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Abstract
Obesity is a costly and growing health concern for the modern world and puts individuals at increased risk for chronic illnesses. Although obesity is associated with many detrimental peripheral effects, food over consumption is centrally mediated. The hypothalamus and brainstem regions control homeostatic food intake, while hedonic food intake is mainly controlled by the central reward system. Palatable foods are rewarding and over ride the homeostatic system and cause over consumption. Foods high in sugar and fat acutely activate dopamine neurons in the classical reward pathway consisting of the ventral tegmental area projecting to the nucleus accumbens and prefrontal cortex. Chronic intake of palatable foods is associated with neuroadaptations that can lead to behavioral changes and further over consumption. This dissertation characterizes the behavioral, transcriptional, and circuitry changes in the central dopamine system after chronic high fat diet and high fat withdrawal in male and female mice. Four models of diet-induced obesity were examined. Chapters 2 and 3 examined diet induced obesity models and standard chow intervention in different age groups in order to reveal a developmental period sensitive to programming effects. We discovered that both age and sex were critical factors in the development and the reversal of neuroadaptations seen in obesity. Early life nutrition is particularly important to the developing brain and overnutrition during this time period leads to epigenetic changes that may contribute to the neuroadaptations that persist after intervention. In chapter 4, we examined exercise as an intervention to prevent the neuroadaptations and neuroinflammation associated with high fat diet intake. We discovered that although exercise had a beneficial effect of weight gain, it was not able to reverse reward dysfunction or neuroinflammation in all cases of high fat intake. It is possible that the neuroadaptations that occur after high fat consumption contribute to the difficulty individuals have with weight loss. Understanding how age and sex impact brain and behavior in the high fat withdrawal stage will have implication for obesity management and interventions. We know now that the brain of an individual is markedly different than the brain of a lean individual and these differences can predispose one to overconsumption during dietary intervention. Since both pharmacological and behavioral therapies are often combined with diet replacement, understanding the brain during this switch and factors that can raise adherence rates will help the success of therapies in the future.

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ABSTRACT

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Jesse Lea Carlin

Teresa M Reyes, Ph.D.

Obesity is a costly and growing health concern for the modern world and puts individuals at increased risk for chronic illnesses. Although obesity is associated with many detrimental peripheral effects, food over consumption is centrally mediated. The hypothalamus and brainstem regions control homeostatic food intake, while hedonic food intake is mainly controlled by the central reward system. Palatable foods are rewarding and over ride the homeostatic system and cause over consumption. Foods high in sugar and fat acutely activate dopamine neurons in the classical reward pathway consisting of the ventral tegmental area projecting to the nucleus accumbens and prefrontal cortex. Chronic intake of palatable foods is associated with neuroadaptations that can lead to behavioral changes and further over consumption. This dissertation characterizes the behavioral, transcriptional, and circuitry changes in the central dopamine system after chronic high fat diet and high fat withdrawal in male and female mice. Four models of diet-induced obesity were examined. Chapters 2 and 3 examined diet induced obesity models and standard chow intervention in different age groups in order to reveal a developmental period sensitive to programming effects. We discovered that both age and sex were critical factors in the development and the reversal of neuroadaptations seen in obesity. Early life nutrition is particularly important to the developing brain and overnutrition during this time period leads to epigenetic changes that may contribute to the neuroadaptations that persist after intervention. In chapter 4,

we examined exercise as an intervention to prevent the neuroadaptations and neuroinflammation associated with high fat diet intake. We discovered that although exercise had a beneficial effect of weight gain, it was not able to reverse reward dysfunction or neuroinflammation in all cases of high fat intake. It is possible that the neuroadaptations that occur after high fat consumption contribute to the difficulty individuals have with weight loss. Understanding how age and sex impact brain and behavior in the high fat withdrawal stage will have implication for obesity management and interventions. We know now that the brain of an individual is markedly different than the brain of a lean individual and these differences can predispose one to overconsumption during dietary intervention. Since both pharmacological and behavioral therapies are often combined with diet replacement, understanding the brain during this switch and factors that can raise adherence rates will help the success of therapies in the future.

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CHAPTER 1: General Introduction

The current statistics on obesity in the U.S are staggering with 34.4% of adult males and 36.1% of adult females considered obese in 2010 (Ogen et al., 2012). The obesity trend in adolescents is just as dire; 23.9 million children ages 5-19 are overweight or obese and the epidemic continues to grow (Go et al., 2013). Both genetic and environmental factors contribute to the development of obesity. Population studies have revealed specific gene mutations to cause monogenic forms of human obesity (Farooqi et al., 2005), and monozygotic twin studies reveal adiposity levels to be strongly heritable (Stunkard et al., 1986; Maes et al., 1997). However, those single gene mutations are very rare and human genetics are very stable. Therefore, it is unlikely that the current obesity epidemic is explained by mutations or gene x environment interactions alone. The most drastic change for humans has occurred in the environment. The broad availability of energy dense foods and the decrease in daily activity has most Americans living in an environment that drives obesity and makes weight loss extremely difficult. The obesogenic environment affects the individual as early as the fetal stage. Over nutrition or poor nutrition during early life development can put one on a trajectory toward obesity in adulthood. Body weight and eating patterns early in life are extremely important in setting the risk for development of obesity and the ability to lose weight. In fact, it has recently been shown that establishment of obesity at age 5 predicts obesity at age 18 (Cunningham et al., 2014). Sex is another factor that plays a role in an individual's obesity risk. Women tend to have higher rates of obesity compared to males (Go et al., 2013). Women have higher overall body fat content as well as additional distribution to the breast, buttocks, and lower body (Martin et al., 2013). Not only do women have a different physical presentation of obesity, women also

have different behaviors toward palatable foods that could put them at increased risk for overconsumption. Women rate food images as more pleasant, they have more negative feelings in the hunger state, and have a higher fMRI response to food cues compared to males (Geliebter et al., 2013). In an effort to better treat and/or prevent obesity, an understanding of how sex and early life experiences interact to affect obesity risk is required.

Central Acting Interventions for Obesity

Approaches to the treatment of obesity include dietary intervention, exercising, pharmacological treatments, and gastric bypass surgery. Success rates at weight loss vary enormously between individuals and, in most cases, lost weight is gained back. Although obesity is associated with peripheral effects, such as increased adiposity, high blood pressure, and inflammation, food consumption is controlled centrally. In fact, many of the single gene mutations known to cause obesity do so by failing to produce satiety. The most studied Mendelian inherited obesity genes (leptin, leptin receptor, pro-opiomelanocortin, melanocortin 3 receptor, melanocortin 4 receptor) lie within the central leptin-melanocortin signaling pathway of the hypothalamus and result in human obesity when loss of function occurs from mutation (Montague et al., 1997; Clement et al., 1998; Farooqi et al., 2007; Krude et al., 1998; Yeo et al., 1998). Looking at obesity as a central food intake issue is an important direction for the field and has led to the development of centrally acting drugs to combat adiposity. Clinical drug trials for weight loss have targeted the brain in order to attempt to decrease food intake. Most medications prescribed for obesity regulate satiety through an effect on serotonergic, noradrenergic or dopaminergic receptor systems (Bray et al., 2000, Clapham et al., 2001). Action on these neurotransmitter systems has led to reduced appetite or hunger

and, thus decreased food-seeking behavior. Four centrally acting noradrenergic agents (phentermine, diethylpropion, phendimetrazine, benzphetamine) are currently FDA-approved for management of obesity (Yanovski et al., 2014). These agents reduce appetite by activation of adrenergic and dopaminergic receptors. However, even in the most successful trials, high rates of drop out and weight gain occur at follow-up time points (Ioannides-Demos et al., 2012). There are still many obstacles, including non-CNS effects that make it so difficult to lose weight and need to be overcome during obesity intervention. Age of onset of obesity is one factor not taken into consideration when analyzing clinical weight loss trial data. It is true that weight loss becomes more challenging as one ages, and adults who were obese as children find it even more difficult to lose weight (Cockrell & Skinner, 2008). Identifying the factors that make childhood obesity more resistant to weight loss may lead to changes in pharmacotherapy for this population in the future.

The Obese Brain

Obesity is associated with excessive overeating and preference for palatable, high-fat foods even when body energy stores are high (Sclafani, 2001; Gaillard et al., 2008). There seems to be a mismatch between the caloric needs of the body and what the brain tells the body to consume. Since the brain controls food intake, much research has been done to see how the obese brain has changed in comparison to its lean counterpart. Imaging studies in obese patients have given us insight into the regions possibly involved in the loss of homeostatic food consumption. Combined evidence points to regions involved mainly in reward processing, taste, and attention that are altered in obese patients. Imaging studies in obese patients have detected increased blood flow to the striatum, the orbitalfrontal cortex, and the insula when viewing pictures

of highly palatable foods (Rothemond et al., 2007; Scheinle et al., 2009, Sharmueller et al., 2012). Imaging studies have also revealed diminished functional connectivity between these regions important for reward evaluation (Stoeckel et al., 2009; Kulleman et al., 2011). These changes are thought to lead to deficiencies in evaluating food reward value and contribute to the drive to overeat. In fact, behavioral studies have shown obese individuals to direct their attention to food related stimuli more so than lean individuals (Nijs et al., 2010). This shift in attention and preference may also increase the vulnerability to further over consume food. The shift in attention toward a learned reward is not specific to obesity in general, but has also been seen to occur in addicted individuals and drug cues. Obese patients seem to have an increased central response to food cues, but how does the brain react during actual food consumption?

Interestingly, studies have revealed a decrease in the hedonic response to food intake. Over weight individuals have reduced activation of the striatum in response to ingesting a palatable solution (Stice et al., 2010). Although obese individuals seem to react strongly to food cues, the hedonic response to intake is diminished. Hedonic drive is mediated by the dopamine reward system and is acutely activated by rewards like palatable foods, sex, and drugs of abuse. A decrease in dopamine receptors have been reported in the striatum of obese patients and this has implications for appetite control as well as other reward behaviors (Wang et al., 2001, Steele et al., 2010). Some, but not all, differences in the obese brain reverse after individuals lose weight. Imaging studies have also looked at the post-obese population and have shown insula activation remains elevated after weight loss (DelParigi et al., 2004). On the other hand, experiments have also shown normalization of dopamine receptors after bariatric surgery (Steele et al., 2010). The neurobiological changes that persist after intervention may contribute to the high failure rates and relapse to unhealthy eating habits. It is important to understand if

some kinds obesity interventions can reverse or prevent the brain changes seen in obesity better than other types on intervention.

The previous studies support the theory of reward dysfunction in obesity. Obese individuals seem to be more sensitive to food cues; however, reward activation is diminished during food intake. This would suggest that there is an increased drive to obtain food. However, because patients are less sensitive to the rewarding effects of food, they would therefore consume more in an effort to obtain sufficient reward levels. This has been demonstrated in progressive ratio studies where obese individuals give a higher reinforcing value to food (Epstein et al., 2007; Temple et al., 2008). Future studies are warranted to see if a higher reinforcing value plus diminished reward activation are behind the overconsumption of palatable foods in obese individuals and if there are sub populations of obese patients that may benefit from targeting overconsumption behaviors.

Obesity Interventions

Successful weight loss is extremely difficult for the obese population and is influenced by many factors, such as age of onset and sex. Caloric restriction or "dieting" is very effective for weight loss, but has proven to be very difficult to adhere to in the general population. One reason is caloric restriction induces biological changes that make adherence difficult. The over abundance of highly rewarding, highly palatable foods makes relapse even easier. Dieting produces changes in appetite, notably an increase in hunger (Pasman et al., 1997) and persistent thoughts about eating (Green et al., 2005). Other approaches to the treatment of obesity include, exercise, pharmacological treatments, and gastric bypass surgery. Pharmacological interventions cause a moderate loss in body weight and have many negative side effects.

Pharmaceutical trials focus exclusively on reduction of body mass and normalization of markers of obesity and do not usually assess the state of mind or drive to eat. Gastric bypass surgery is a last line of defense treatment for extreme obesity and is very successful in long-term body weight reduction. Adaptive responses that increase appetite after weight loss do not seem to occur after weight loss induced by bariatric surgeries as they do with typical dieting interventions (Berthoud et al., 2012). Bariatric surgery patients have a diminished desire to eat (Shultes et al., 2010) and exhibit normalization of dopamine receptor D2 (DR2) levels after surgery (Steele et al., 2010; Dunn et al., 2010). However, dieting is still the most common way to attempt weight loss in the obese population. How the dopamine reward system changes after high fat replacement needs to be studied in order to better understand the behavioral changes that either promote or hinder adherence to a diet. Characterizing how dopamine circuitry changes when high fat diet is switched to a low fat diet may help us discover why dieting is so difficult for obese individuals. Additionally, characterizing important factors, such as age and sex that impact the response to high fat withdrawal will be important for analyzing clinical weight loss trial results in the future.

Many factors, such as stress, can make caloric restriction difficult and cause return to high fat food consumption. High-fat diet removal elicits palatable food cravings and exposure to foods cues and/or stressful experiences can cause relapse (Nair et al., 2009). Many kinds of stress, such as physical, psychosocial, and pharmacological stressors can stimulate palatable food seeking and return to unhealthy eating habits (Grilo et al., 1989; Ghitza et al., 2006; Nair et al., 2011). This is comparable to what is seen in drug addiction. After extinction of the drug reward, different types of stressors can reinstate drug seeking (Hyman et al., 2006). Many researchers have noted similarities between drug withdrawal and palatable food removal. It has even been

documented that removal of a high fat/high sugar diet causes withdrawal symptoms similar to opiate withdrawal (Avena et al., 2008a). The mechanisms behind stress-induced reinstatement of palatable food seeking are still being examined. Nair et al., (2011) have discovered that blocking dopamine receptors in the prefrontal cortex can inhibit stress-induced reinstatement of food seeking. Ghitza et al. (2006) discovered a similar role for CFR1 receptors in the amygdala. By blocking activation of CRF1, food seeking is not reinstated by a pharmacological stressor. Food cues also have the ability, like stress, to trigger relapse to palatable food seeking. The reinstatement by food cues is likely to be mediated by additional mechanisms and brain regions than reinstatement by stressors. Priming with drugs that increase dopamine, such as cocaine or amphetamine works to reinstate palatable food seeking as well as priming with food or a cue that predicts palatable food (Ghitza et al., 2007; Keiflin et al., 2008; Odum & Shahan, 2004). One factor to consider when interpreting these results is many of these studies were performed in lean rodent models. There are many neurobiological changes that occur after diet induced obesity and research on palatable food reinstatement after acute high fat withdrawal or long term high fat withdrawal need to be examined.

We have seen that stressors can be a trigger to cause increased intake of palatable foods. However, can removal of the palatable food be a stressor in itself? Little is known about the consumption of palatable foods following withdrawal from chronic HFD and the reward-relevant neurobehavioral changes that may occur. We know that food restriction in lean animals causes stress and increases administration of drugs of abuse like cocaine and amphetamine (Carroll and Meisch, 1980; Stuber et al., 2002). Other studies have confirmed a strong relationship with food restriction and the increase in the rewarding properties of drugs such as amphetamine (Takahashi et al., 1978), heroin (Oei et al., 1980) and cocaine (Carroll et al., 1981). It is unlikely however,

that food restriction in lean animals is similar to withdrawal of a high fat diet in obese animals, given what is known about the differences in reward relevant circuitry in the brains of lean and obese animals. High fat diet withdrawal has also been shown to increase responding for sucrose in obesity prone rats (Pickering et al., 2009). This suggests that the propensity to relapse may be stronger in some individuals than others. Further, we know from Zorilla lab and colleagues (Cottone et al., 2008a; 2008b; 2009) that intermittent access to a high fat diet causes anxiety behavior and increases responding to palatable food reward in rodents. Others have found that an acute withdrawal (only 24 hours) causes anxiety behavior in an open field test (Teegarden et al., 2007). Withdrawal from a high fat diet can increase basal corticosterone levels (Sharma et al., 2013) but has no effect on stress induced cort levels (Pickering et al., 2009) and changes in CRF levels in the amygdala (Teegarden et al., 2007). Removal of other rewarding substances, such as sucrose, can also be a stressor and cause an increase in anxiety-like behaviors (Avena et al., 2008b). Body weight loss goes hand in hand with high fat diet removal. It is possible that body weight loss can cause an activation of the body's stress system as well (Bailey et al., 2004). Looking at a time point where high adiposity is still present but the diet has been withdrawn can be a helpful way to control for the stress of high body weight loss. Although changes in CRF1 levels were not seen in the amygdala after high fat withdrawal in a diet induced obese model (Sharma et al., 2013), it is still possible that activation of stress circuitry after high fat diet removal can contribute to reward behavior observed after high fat diet removal.

Homeostatic Regulation of Food Intake

The hypothalamic and mesolimbic dopamine systems participate in the integration and control of food intake. Dopamine acting in the hypothalamus is important for basal food intake and influences feeding frequency and volume (Meguid, et al., 2000). For example, dopamine is released in the lateral hypothalamic area in response to feeding and normalizes after meal termination (Yang et al., 1996). Dopamine release in the hypothalamus leads to activation of neuropeptides involved in both food intake stimulation (e.g. melanin concentrating hormone (MCH), neuropeptide Y (NPY), Agouti related peptide (AgRP), galanin, and orexin) and food intake inhibition (e.g., a-melanocyte-stimulating hormone (MSH), cocaine and amphetamine-regulated transcript (CART), and corticotrophin-releasing factor (CRF)) depending on the complex metabolic conditions occurring in the body. The basic circuitry of the hypothalamus and brain stem is under multiple points of regulation and has the ability to sense macronutrients, hormones, gut signals, and energy stores to control energy metabolism and food intake behavior in order to maintain homeostasis. There are two main sources of circulating nutrients: food intake, and production of glucose and lipids by the liver. Increased availability of macronutrients such as glucose and lipids activates sensing pathways in the brain, either directly through metabolic signals (e.g., fatty acids), or indirectly, through stimulation of insulin and leptin biosynthesis and secretion. Leptin is synthesized by white adipose tissue, and its level increases in proportion to fat mass. Among its many actions, high levels of leptin potently suppress food intake and stimulate metabolic processes to dissipate excessive energy stores. The activation of brain efferent pathways in turn suppresses food intake and hepatic output of glucose and lipids. (Schwartz et al., 1996; Obici et al., 2002; Loftus et al. 2000; and reviewed in Lam et al., 2005). In obesity, however, this feeding circuit is altered and palatable foods are consumed after energy requirements are met. It has been confirmed that

overconsumption of palatable foods and obesity can eliminate the ability of intracerebroventricular (icv) administration of insulin to reduce food intake (Posey et al., 2009). The hypothalamus also becomes insensitive to leptin signals and there is a decrease in AGRP signaling to the mesolimbic pathway (Varela & Horvath, 2012). How the high fat diet impairs the negative feedback system is unclear. One hypothesis is that high fat diet causes neuroinflammation in the hypothalamus that impairs insulin sensitivity and the signal to stop eating (DeSouza et al., 2005). Palatable foods that drive obesity are often consumed after energy needs are met. It is thought that other brain regions, such as the mesolimbic dopamine area, control the intake of energy dense foods and this is one area pharmacotherapy can target in obese patients in the future.

The Dopamine Reward Pathway

The modern environment is characterized by the availability of highly palatable, energy dense foods as well as powerful food cues. High fat and high sugar foods activate the mesolimbic dopamine system to drive food intake even after energy requirements have already been met (Berthoud et al., 2007; Stratford & Kelly, 1999). Dopamine neurons in the mesolimbic pathway originate in the ventral tegmental area and have been implicated in the reinforcing and motivational aspects of food intake (Ikemoto & Panksep, 1996; Brennan et al., 2001; Ishiwari et al., 2004). Dopamine neurons are found in the VTA and project to the NAC to form the classic reward pathway studied in drug addiction. The dopamine neurons in the VTA also innervate several regions of the prefrontal cortex (PFC), central amygdala, basolateral amygdala (BLA) and hippocampus. All the reward related regions are inter-connected and synapse back on each other in complex ways. The VTA–NAc circuit is crucial for the recognition and consumption of rewards (Koob et al., 2008). DA in these regions is synthesized from

tyrosine, through the conversion of l-tyrosine to l-dihydroxyphenylalanine (l-DOPA) by the rate-limiting enzyme tyrosine hydroxylase (TH). DOPA is subsequently converted to DA by the enzyme l-aromatic amino acid decarboxylase. DA is then transported from the cytoplasm to storage vesicles via the synaptic vesicular monoamine transporter (VMAT). After DA is released into the synapse, the plasma membrane DA transporter (DAT) can transports DA in and out of the terminal depending the concentration gradient and other factors (Amara and Kuhar, 1993). The conversion of DA to HVA occurs extra-neuronally by the enzyme catechol-*O*-methyltransferase (COMT) (Feldman et al., 1997).

Receptors that translate dopamine release into a signal are found in projecting regions NAc, PFC, amygdala, and other regions. DA receptors are classified into two subfamilies, D1- and D2-like receptors (Brown and Makman, 1972; Kebabian and Calne, 1979), based on their pharmacological and functional properties. The D1-like receptors are dopamine receptor D1 (DR1) and D5 (DR5) (Sunahara et al., 1990 and Sunahara et al., 1991), whereas the D2-like receptors are dopamine receptor D2 (DR2), D3 (DR3), and D4 (DR4) (Sokoloff et al., 1990) based on their respective gene sequences. Dopamine receptors D1 and D5 are coupled to adenylyl cyclase through G protein G_s, while dopamine D2, D3, and D4 receptors inhibit adenylyl cyclase by coupling to $G_{i/o}$. The D1-like and D2-like receptors are both present in all dopamine-containing regions of the rodent brain (Meador-Woodruff, 1994). Higher levels of DR2 mRNA are detected in the substantia nigra and VTA. DR3 display a much more restricted pattern of distribution and are mainly found in limbic regions (Levesque et al., 1992). D1R knock out (KO) mice show normal appearance and no obvious neurological defects, but exhibit growth retardation and low survival after weaning (Drago et al., 1994). Midbrain levels of dopamine are elevated in D1R KO mice, suggesting a compensatory increase in dopamine synthesis and/or release following inactivation of the receptor (El-Ghundi et

al., 1998). D2R KO mice exhibit significantly lower levels of locomotor activity than controls (Palmer et al., 2003) but total dopamine levels are not altered in D2R KO mice, suggesting D2R does not exert strong tonic control of dopamine activity (Benoit-Marand et al., 2001). On the post-synaptic side, the dopamine signal is transduced after activation of DR1 by the actions of Protein phosphatase 1 regulatory subunit 1B (DARPP-32). DARPP-32 is phosphorylated by cAMP-dependent protein kinase (PKA) and cyclin-dependent kinase 5 (Cdk5) on threonine residues, thr34 and thr75, resulting in inhibition of protein phosphatase-1. Additionally, DARPP32 dephosphorylation at serine residue 97 has also been observed and this modification appears to be important in both addiction and food intake behaviors (Stipanovich et al., 2008). Dopamine signaling elicited by drugs of abuse or palatable foods also increases the activity of ΔFOSB, cyclic AMP-responsive element binding protein (CREB), and cyclin-dependent kinase 5 (CDK5) pathways in medium spiny neurons (Joressen et al., 2007; Carlezon et al., 1998; Bibb et al., 2001). These proteins play a prominent role in the synaptic plasticity seen after chronic drug intake and could possibly regulate plasticity after other rewarding substances, such as palatable food.

Hedonic Eating

Energy dense foods are widely available and there are many aspects of modern society tempting us to eat. Wanting' and 'liking' reactions are generated by neural systems after the intake of sweet, palatable foods. Drive to eat palatable foods is still present even when the individual is sated. For example, in humans, pleasure ratings for chocolate never hit zero even after consumption of two whole bars (Lemmons et al., 2009; Small et al, 2001). The "always room for dessert" phenomenon is also seen in mice who can consume 10% of their body weight in milk and have their drive for

"sweetness" never completely diminish (Berridge et al., 1991). This suggests the ability of certain foods to override homeostatic stop signals and drive over consumption. The "wanting" and "liking" of palatable foods is regulated by dopamine and opioid neurotransmitters in the reward regions. Palatable food activates "hedonic hot spots" in the reward system, specifically the nucleus accumbens, ventral pallidum, amygdala, obitofrontal cortex, anterior cingulate cortex and the anterior insula cortex (de Araujo et al., 2003; Aldridge & Berridge, 2010; Kringelbach et al., 2004; Small et al., 2003). Consumption of palatable foods increases dopamine concentrations in the NAc (Bassarero & DiChiara, 1999). Dopamine in this region is thought to coordinate the action of food intake, increase arousal, and conditioned learning in order to obtain the food reward. Increases in dopamine, as seen in dopamine transporter (DAT) knockout mice, produce elevated "wanting" for sweet foods (Pencina et al., 2003). Palatable food is a powerful motivating force. Rats prefer palatable solutions to cocaine infusions (Lenior et al., 2007), and will voluntarily expose themselves to a noxious environment or pain to obtain high fat food over standard chow (Cabanac and Johnson, 1983; Foo and Mason, 2005). In fact, DA is necessary for the motivation to consume food (Ungerstedt , 1971; Zhou & Palmiter, 1995) and DA-deficient mice from tyrosine hydroxylase gene knockout are hypophagic and eventually die of starvation unless DA is restored to the striatum (Szczypka et al., 2001). Dopamine also controls food choice. Dopamine agonists have been shown to enhance preference for and the motivation to obtain fatty foods in rodents (Thanos et al., 2011, Cooper et al., 2006). These taken together show that palatable foods activate the dopamine reward system acutely and cause overconsumption of calories. How exactly palatable food elicits the release of dopamine is currently unclear. It has been demonstrated, however, that the reward effect is independent of taste receptor activation (Ferriera et al., 2012; De Araujo et al., 2008).

Further investigation is needed to see what role post-ingestive factors, such as fat sensing in the gut, have in reward signaling in the brain.

Interestingly, mutant mice lacking dopamine are still able register the hedonic impact and show preferences, and learning to work for a palatable sweet reward. This suggests other neurotransmitters have a role in maintaining hedonic intake of food. In fact, a number of investigators found opioid or cannabinoid receptor activation in the nucleus accumbens to stimulate appetite by enhancing the 'liking' for the perceived palatability of food (Bodnar et al., 2005; Cooper, 2004; Kelly et al., 2002; Zhang & Kelley, 2000). Opioids in the nucleus accumbens shell are involved with the "wanting" aspect of palatable food intake through the modulating effect they have on dopamine release. Activation of mu opioid receptors indirectly dis-inhibit dopamine neurons and thereby increase dopamine release. For example, DAMGO, a mu-opioid receptor agonist, more than doubled the amount of food intake when administered centrally to rats (Pencina & Berridge, 2005). Moreover, anandamide, an endocannabinoid that likely acts in the brain by stimulating the CB1 type of cannabinoid receptor, has been shown to act in the nucleus accumbens to magnify the impact of sucrose taste (Mahler et al., 2007). There has also been evidence for additional hedonic neurotransmitters acting in the nucleus accumbens to enhance "liking" of palatable foods. The ventral pallidum/nucleus accumbens region receives orexin inputs from the hypothalamus (Nixon & Smale, 2007) and orexin in this region can enhance 'liking' for sweet rewards (Harris et al., 2005). While more studies are needed, it is possible that dopamine dysfunction in the reward system seen in obesity can allow these other neurotransmitters to have a larger role and promote over consumption of palatable foods.

Neuroadaptations in DA system after Chronic HFD

Research in both human and animals suggests that long-term intake of palatable foods leads to neuroadaptations in the dopamine reward system that could have implications for obesity treatments and other reward behaviors. Several lines of evidence support the hypothesis of dysregulated dopamine function in obesity. Chronic consumption of a high fat diet is associated with a state of reward hypofunction and imaging studies reveal blunted activation of brain reward regions during consumption of palatable foods in obese individuals (Stice et al., 2008). Other human studies have shown a down regulation of dopamine D2 receptor in obese individuals (Wang et al., 2001). This is consistent with obesity being overrepresented in Taq1AA1 allele carriers that affects dopamine D2 receptor (DR2) expression (Blum et al., 1996, Nobel et al., 1991). Down regulation of DR2 could potentially be involved in the increased craving and increased reward seeking that is associated with the obese population (Blum et al., 2008; Downs et al., 2009). Down regulation of DR2 has previously been associated with many other disorders that are involved with dysregulation of motivation such as addiction, and attention deficit hyperactivity disorder (Volkow et al., 2011a; Volkow et al., 2011b).

Obesity prone rats have shown increases in extracellular dopamine in the striatum (Narayanaswami et al., 2005). However, when animals become obese we see a decrease in dopaminergic tone. Animal models of diet-induced obesity (DIO) exhibit lower basal extracellular dopamine levels in the nucleus accumbens (NAc) and the ventral tegmental area (VTA) (Geiger et al., 2007; Geiger et al., 2009; Cone et al., 2010; Rada et al., 2010). Diet induced obesity models have lower dopamine turnover (Davis et al., 2008), decreased dopamine release (York et al. 2010), and reduces DA clearance

(Speed et al., 2011) compared to standard chow controls in the nucleus accumbens. Genes involved in dopamine uptake and metabolism in addition to dopamine receptors DR1 and DR2 have been reported to decrease expression in obese rodent models (Alsio et al., 2010, Huang et al., 2005). Protein levels of both DAT and DR2 protein levels are also diminished after high fat diet intake (Huang et al., 2006; South & Huang, 2008; Johnson & Kenny, 2010). Decreases in sucrose preference (Vucetic et al., 2012, Carlin et al., 2013), a decrease in response for a sucrose pellet (Davis et al., 2008) and a decrease response to food reward (Corwin et al., 2011; Cottone et al., 2008; Johnson and Kenny, 2010) are associated with the dopaminergic protein changes after high fat intake. Changes in DR2 expression may impact the animal's willingness to work for a goal (Trifilieff et al., 2013) and an animal's sensitivity to dopamine agonists. For example, rats eating high fat chow are more sensitive to quinpirole-induced yawning (D2 and D3 receptor-mediated; Baladi and France, 2010).

Neuroadaptations in Leptin, Insulin, Ghrelin in the VTA

The previous body of literature supports altered dopamine function in obesity and suggests a decreased sensitivity to reward in the obese population. Whether the high fat diet is directly affecting dopamine metabolism and release at the molecular level or other adjacent neurotransmitter systems are indirectly changing VTA activity is currently unclear. It is possible that hormones involved with energy homeostasis may play a role in natural reward intake and changes in signaling may disrupt dopamine and lead to hedonic overeating. It is known that insulin, leptin, and ghrelin can decrease food reward behaviors and modulate the function of neurotransmitter systems that mediate food reward. Insulin receptors are expressed in brain regions that are rich in DA neurons and studies have shown co-expression of insulin receptor with tyrosine hydroxylase

(Figlewicz et al., 2003). This suggests an interaction between energy sensing and DA systems occurring in the reward regions. Manipulations of insulin concentrations significantly affect DA synthesis, turnover, and signaling in reward regions (Kwok and Juorio, 1986; Lim et al., 1994; Lozovsky et al., 1981; Saller, 1984). For example, acute injection of insulin into the brain enhances expression and activity of dopamine transporter (DAT) (Carvelli et al., 2002; Figlewicz et al., 1994). High fat diet consumption causes both peripheral and central insulin resistance over time. Because insulin regulates DA systems, it is likely that decreases in insulin signaling could contribute to the changes seen in dopamine circuitry and reward behaviors in obesity (Daws et al., 2011; Niswender et al., 2011). Leptin levels are associated with total adipose fat stores and may directly or indirectly regulate dopamine dysfunction in obesity. Leptin receptors are also found in the VTA and are downregulated in DIO animals (Figlewicz et al., 2003; Fulton et al., 2006; Blendy et al., 2005). Leptin has been shown to modulate response for rewarding brain stimulation, and modulate conditioning for high fat food (Fulton et al., 2000 and Fulton et al., 2004). Leptin knockout mice (ob/ob mice) have a significant impairment of DA release and tyrosine hydroxylase capacity in the NAc (Fulton et al., 2006).

Ghrelin is a gut-derived hormone that is associated with food-deprivation and ghrelin receptors are also distributed in the ventral tegmental area and co-express with tyrosine hydroxylase (Mitchell et al., 2001). Ghrelin is involved in appetitive actions and the food seeking response (Naleid et al., 2005) possibly through the action of increasing VTA action potentials and the release of dopamine in the nucleus accumbens (Abizaid et al., 2006; Abizaid, 2009). It is thought that ghrelin signaling is altered in obese individuals and affects dopamine dependent behaviors such as novelty seeking (Savage et al., 2014) and high fat/high sugar intake (Menzie et al., 2013). One common

downstream signaling mechanism in the VTA for these energy related hormones is PI3Kinase. Studies have identified that insulin and leptin increase PI3kinase activity in the VTA (Figlewicz et al., 2007) and increase JAK-STAT phosphorylation, which is necessary for the decrease in feeding response (Morton et al., 2009). One potential cellular target for PI3Kinase is the DAT. It has been shown that insulin regulates the expression and activity of DAT through PI3Kinase (Garcia et al., 2005; Vaughan et al., 1997) and diabetic models lacking insulin show DAT deficits (Figlewicz et al., 1994; Sevak et al., 2008). This implies that insulin resistance in obesity may alter DAT expression and activity and therefore alter DA signaling and reward seeking behavior. Energy related hormones are perfectly poised to regulate reward behavior and palatable food intake. If overweight individuals are less sensitive to homeostatic signals and therefore less sensitive to natural rewards they may consume more in an effort to obtain a larger positive effect. This is similar to the phenomenon of "tolerance" in addicted individuals. Moreover, altered sensitivity to natural rewards may also mean altered sensitivity to drugs of abuse that activate the same reward pathways.

The Neurobiological Overlaps of Addiction and Obesity Pathways

Dopamine is the key neurotransmitter involved in drug abuse as well as food intake. It is important to understand factors, such as obesity, that might alter sensitivity to the behavioral effects of drugs acting directly or indirectly at DA receptors. The repeated stimulation of dopamine reward pathways leads to adaptations in neurotransmitters and reward circuitry that may lead to increases in compulsive behaviors affecting both food and drug intake (Volkow & Li, 2004). Human studies reveal that obese individuals display decreased propensity to use recreational drugs and a decreased prevalence of substance abuse disorders (Simon et al., 2006; Warren et al., 2006; Bluml et al., 2012).

Additionally, obese individuals have been shown to self-administer less nicotine and demonstrated diminished hedonic drive toward nicotine cigarettes than their lean counterparts (Blendy et al., 2005). In one demographic study being “obese” or extremely obese” has been associated with an increase risk of alcohol abuse disorder as well as other mood and anxiety disorders (Petry et al., 2008). Research in both fields confirms the mechanisms behind addiction and overconsumption of palatable food converge on the same molecular targets in the reward system. One neuroadaptation found in both obesity and addiction is the decreased expression of DR2. Positron emission tomography has revealed decreased DR2 in the striatum of addicted individuals that persists well after drug withdrawal (reviewed in Volkow et al., 2009). This finding has also been replicated in preclinical rodent studies given chronic exposure to drugs of abuse (Thanos et al., 2007; Nader et al., 2006; Volkow et al., 2001). Down regulation of both DR2 and DR1 expressing neurons in the striatum that mediate the direct striatal pathway enhances the sensitivity to drugs of abuse (Fergusen et al., 2010; Hideka et al., 2010). Dysregulated dopamine signaling in this region is likely to contribute to the compulsive and impulsive drug intake seen in addiction (Goldstein et al., 2002).

High impulsivity may underlie the inability of obese individuals to resist excessive eating. Both addicted and obese individuals show impairment in PFC and OFC regions of the brain that control reward and impulsivity (Volkow et al., 2001; Volkow et al., 1993; Volkow et al., 2008). Moreover, decreased D2R striatal expression and signaling has been detected among obese individuals (Geiger et al. 2009; Wang et al., 2001, de Weijer et al. 2012) and in rodents given a high fat diet (Johnson & Kenny, 2010). Other studies have shown overeating palatable foods can produce behavioral and neurochemical signs that resemble dependence in laboratory animal models such as compulsivity, adaptation, and sensitization to food (Avena et al., 2008, Corwin et al.,

2011 and Volkow et al., 2011c). When rewarding substances target the same downstream signaling mechanisms they have the ability to cross sensitize or cause tolerance towards one another. For example, eating high fat chow increases sensitivity of drugs acting at both D3 and D2 receptors (Baladi et al., 201, Corwin et al., 2011). High fat diet intake also leads to increased sensitivity to cocaine (Baladi et al., 2012; Shumsky et al., 1997) and methamphetamine (McGuire et al., 2011).

Addictive drugs act on known receptors to increase dopamine release or dopamine levels in the nucleus accumbens. It is not yet fully elucidated how palatable foods increase dopamine in nucleus accumbens, but there are some downstream molecular targets that are affected in both palatable food consumption and addiction that could give us some hints. Dopamine signaling in the accumbens modulates the activity of ΔFOSB, cyclic AMP-responsive element binding protein (CREB), DARPP32 and CDK5 signaling pathways in medium spiny neurons, and influences the rewarding properties of both food and addictive drugs (Kenny, 2011). One example of this is ΔFOSB. ΔFOSB is increased in the striatum of rodents given a high fat or high sucrose diet and is associated with the motivation to obtain palatable food (Christiansen et al., 2008; Teegarden & Bale, 2007). Cocaine and other drugs of abuse increase the expression of ΔFOSB throughout the striatum, more specifically in the dopamine receptor D1 and dynorphin-expressing medium spiny neurons in the direct pathway (Muller et al., 2005). The overlap of addiction and food intake pathways can also be seen with peptides involved in homeostatic feeding and is reviewed extensively in (Volkow et al., 2013). Orexigenic peptides ghrelin, orexin, melanocortin, and neuropeptide Y are found to affect reward regions and modulate reward behavior such as alcohol intake (Leggio et al., 2011; Gilpin et al., 2012), conditioned reward learning for cocaine (James et al., 2011) and morphine self administration and conditioned place

preference (Harris et al., 2007; Proudnikov et al., 2008). Anorexigenic peptides also act in reward regions of the VTA, NAc, and amygdala to modulate reward behavior. Glucagon-like peptide-1 (GLP-1), cholecystokinin (CCK), peptide YY (PYY), galanin, cocaine-and amphetamine-regulated transcript (CART), corticotropin-releasing hormone (CRH), and oxytocin have been shown to affect behaviors towards amphetamines (Erreger et al., 2012; Vaccarino et al., 1994; Crawley et al., 1985; Beinfeld et al., 2003), alcohol (Barson et al., 2011; Le Strat et al., 2012), and cocaine (Cippitelli et al., 2012; Upadhya et al., 2012). The regulation of homeostatic food intake, palatable foods, and other natural rewards all converge on the dopamine neurons in the ventral tegmental area. The dopamine reward system is hijacked by unnatural rewards (drugs of abuse) and neuroadaptations that occur in addiction to drugs of abuse are similar to those seen in obesity. The combined results suggest that both obese and drug-addicted individuals suffer from overlapping impairments in dopaminergic pathways that lead to overconsumption and impulsivity.

Another neurotransmitter system that is involved in both palatable food intake and reward is the central opioid system. Both neurotransmitter systems are activated in concert and are dependent on each other. For example, opioids increases intake of palatable foods in sated animals and this depends on the expression of dopamine receptor D2 (Hayward et al., 2007). Further, opioid receptors are distributed throughout regions important for food and drug reward (Olszewski, et al., 2007) and mu opioid receptor activation increases dopamine release (Bontempi et al., 1997; Horner et al., 2012; Steiner et al., 1999). Mu opioid receptor (MOR) expression is altered by high fat diet at different time periods. Chronic high fat diet in adulthood decreases MOR expression (Vucetic et al., 2011; Ong et al., 2013; Blendy et al., 2005). Conversely, high fat diet during pregnancy increases expression of MOR in reward regions of the offspring

(Vucetic et al., 2010; Ong et al., 2011; Grissom et al., 2013; Carlin et al., 2013b). It is yet to be discovered how high fat diet regulated the expression of MOR, however, studies out of our lab point to possible recruitment of epigenetic markers (Vucetic et al 2010; Grissom et al., 2013). It seems that changes in opioid signaling could play a role in the reward dysfunction of obesity either by amplifying the effects of dopamine dysregulation or altering the palatability of food. Endogenous opioids code the palatability of food in the brain and neuroadaptations in this system could have implications in overconsumption of food in obesity. If MOR is down regulated in obesity, it could be a homeostatic mechanism to reduce the drive to eat and the dopamine dysfunction just happens to over ride that effect. Conversely, down regulation of MOR can add to the hypo reward seen in obesity which could further drive palatable food intake in order to normalize reward levels in the brain. Further experiments are needed to answer how endogenous opioids are altered alongside dopamine and if these neuroadaptations reverse after standard chow replacement.

Sex Differences in the Dopamine Reward System and Obesity

Sex differences are found in both the central dopamine system and in the presentation of obesity. The overlapping dopamine pathways seen in obesity and drug addiction are also highly influenced by sex and sex hormones. We see that women are more susceptible to developing eating disorders and obesity compared to men (Hoek et al., 2003; Berghöfer et al., 2008; Keel et al., 2007). Women also have lower success rates at dieting then men. This could be due to their more intense interaction with palatable foods. Women tend to show increased brain activity in the prefrontal cortex and OFC after ingestion of palatable liquids compared to men and this has been associated with decreased inhibitory control when it comes to food intake (Del Parigi et

al., 2002). This difference in inhibitory control could underlie the lower success rates when losing weight with dieting (Appelhans et al., 2011). Studies have shown sex hormones directly influence food intake, body weight, and fat distribution. Preclinical studies in rodents have shown an opposite effect of what we see in humans. Male mice are more vulnerable to HFD-induced weight gain (Martin et al., 2007) and this sex difference can be attributed to the positive metabolic effects of estrogen in females (Heine et al., 2000). Further studies are needed to see why this protective effect is absent in the human population, which is clearly a more complicated issue than what is observed in rodent. Sex hormones also have an effect in the brain and can modulate central leptin signaling. Female rats are more sensitive to the anorexic effects of leptin than male rats (Clegg et al., 2003). Given the gender differences in obesity and the high rate of failure in weight loss therapeutics, examining overconsumption and dopamine circuitry in females would be an extremely important contribution to the field.

Sex differences are also present in the incidence rates of psychiatric disorders. For example, major depression, which involves the disruption in the dopamine reward system, is twice as common in females then in males (Marcus et al., 2005). Women also experience addiction to drugs of abuse differently than men. Female drug users tend to be more sensitive to cocaine-conditioned stimuli and report more intense cravings (Elman et al., 2001; Robbins et al., 1999). Sex differences persist when examining the central reward system in rodents. Female rats develop cocaine CPP after fewer conditioning sessions and with lower doses compared to males (Parylak et al., 2008). This suggests that females are more sensitive to the rewarding effects of drugs acting the dopamine system and this could be behind the sex differences seen in addiction incidence rates (Zakharova et al., 2009). Estradiol has the ability to modulate dopamine at the molecular level. In fact, estradiol rapidly increases dopamine efflux in

the nucleus accumbens (Becker, 1999) and in cell culture (Alyea et al., 2008). Female gonadal steroids inhibit DA neurotransmitter turnover in prefrontal regions and ventral tegmental area (Handa et al., 1997) and regulate the reuptake of DA in regions involved in satiety and motivation depending on estrus cycle (Thompson et al., 1997).

Experiments looking at sex differences have added richness to the addiction field. Studies in the field of neuroscience of obesity often do not include females in the analysis. This is potentially excluding 50% of the population that will receive the pharmacotherapies that come out of these studies. Examining how neuroadaptations to chronic high fat diet and their reversibility are influenced by sex is an important step in developing proper drug targets to combat obesity.

Consequences of Early Life Nutrition on Obesity and Brain Development

Age is another critical factor to consider when studying obesity and the brain. There are important times during development in which the brain seems to be particularly sensitive to the environment. Changes in the environment during critical time periods can alter the trajectory of development and affect the health of the adult in the future. We see this sensitive time period come into play when setting adulthood body weight. Obese children are more likely to become obese adults (Serdula et al., 1993) and obese children will find it more difficult to lose weight when they become adults (Cockrell-Skinner et al., 2010). It seems this critical time period is set before age five in humans. A recent study showed that obesity by age 5 could predict future obesity at age 18 (Cunningham et al., 2014). At first glance, childhood obesity is similar to adult obesity in that it is caused by increase food intake, sedentary lifestyle, complex interaction between over 250 obesity-associated genes and early life factors (Rankinen

et al., 2002). However, childhood obesity can be more detrimental to future health and weight loss goals in the future.

Both genetic makeup and early-life environmental factors play a significant role determining body weight and adiposity. The early life environment begins in utero and is affected by maternal nutrition and health during pregnancy. It seems that the risk for obesity can begin at conception and depend on the in utero nutritional and hormonal environment. Both maternal overnutrition and maternal under nutrition has been associated with obesity later in life (Whitiker & Dietz, 1998; Boney et al., 2005; Ravelli et al., 1976). The in utero environment has the ability to program adulthood appetite, neuroendocrine functioning, and metabolism. Modeling maternal obesity in animals has yielded a wealth of data on altered genes and behaviors that can contribute to behaviors in offspring. The brain is developing throughout gestation; the dopamine reward system circuitry and its control on food intake are not spared from maternal programming. Maternal high fat diet alters hypothalamic neurons that express appetite-regulating neuropeptides, leading to hyperphagia (Plagemann et al., 1999; Muhlhausler et al., 2006) and exacerbated preference for fatty foods in offspring (Vucetic et al., 2010; Bayol et al., 2007). Naef and colleagues (2013a) have shown offspring exposed to high fat diet in utero demonstrate reduced anticipatory response to a food reward. Additionally, these offspring had increased responding for high fat food (Naef et al., 2011). It seems maternal high-fat diet not only increases offspring's risk for obesity, but it also leads to alterations in dopaminergic circuitry. Our lab documented changes in DAT, dopamine receptors DR1 and DR2, DARPP-32, and TH, and mu opioid receptor (MOR) mRNA in reward regions in offspring from a high fat fed dam. These mRNA changes were associated with alterations in DNA methylation at the promoter region of these genes (Vucetic et al., 2010). This is evidence for possible epigenetic mechanisms linking

maternal diet during pregnancy with offspring behavior. These studies show that gestation is a particularly sensitive time period in programming feeding behavior and dopamine circuitry. The brain continues to develop after gestation in humans and even more so in rodents. Another study done by Naef, et al (2010) targeted the last week of gestation as well as the 3 week weaning period. Maternal high fat diet during the later gestation period and weaning led to offspring with weaker amphetamine-induced extracellular NAc DA levels, higher NAc DAT activity, and reduced D_2 receptor mRNA in the VTA. This study demonstrates that post-natal time periods are also sensitive to nutritional insults. The early post-natal period is very important for brain development and sensitive to many types of environmental insults, not just nutrition, which can be associated with dysfunction in adulthood. Disruption of early life environment in humans can lead to increased risk of depression risk (reviewed in Hein et al., 2010, Chapman et al., 2008), inflammation (Danese et al., 2008), glucocorticoid resistance (Carpenter et al., 2004), reduced cortical volume (van Harmelen et al., 2010) and reduced hippocampal volume (Vythilingam et al., 2002) in the adult population. These studies point to the existence of important sensitive time windows in development that can set an individual on a trajectory to dysfunction in adulthood. How early life nutrition interacts with the brain during these critical development periods and leads to adult reward dysfunction and obesity needs to be further explored.

The brain continues to develop from postnatal day one through adolescence. If the environment is particularly damaging during that period, programming of adulthood diseases can occur. “Metabolic imprinting” is a term used to describe how alterations in developing young can predispose individuals to obesity and its associated illnesses. We already examined how gestation was critical for the development of feeding and reward circuits. The weaning period up until adolescence also represents a critical

developmental window with regard to the establishment of metabolic-regulating neural pathways (King et al., 2006). In fact, dietary preferences and feeding behaviors are programmed during childhood (Sato et al.,1991). The projections from the arcuate nucleus to other hypothalamic nuclei regulating energy balance mature by the 8th to 10th day of postnatal life (Grove and Smith, 2003; Grove et al., 2003; Bouret et al., 2004a). These projections are sensitive to both gestational and postnatal overnutrition (Plagemann, 2006; Chang et al., 2008). Additionally, levels of the metabolic hormones leptin and insulin play a role in the development of the hypothalamus and projecting areas during these developmental periods (Bouret et al., 2004b; Frank et al., 2005). Projections from the VTA to the NAc and PFC mature post-natally as well (Antonopoulos et al., 2002; Kalsbeek et al., 1988). These studies demonstrate that early life nutritional insults could have consequences for DA circuitry and DA-mediated behaviors. Early postnatal overnutrition in mice, simulated by a small litter number throughout lactation, predisposes the offspring to adult obesity (Bouret, 2010). Early exposure to a high fat diet for as little as one week was able to alter adult caloric intake and dopamine signaling protein levels (Teegarden et al., 2009). Additionally, high fat during the peripubertal period sensitized the response of dopamine in the NAc to a tail pinch test (Naef et al., 2013b). Finally, high fat feeding was sufficient to disrupt PPI when given during peripubertal period, whilst high fat feeding failed to affect PPI when limited to adulthood (Labouesse et al., 2013). Other brain regions are also affected by high fat diet given early in development, such as the HPA axis, and can also impact feeding and metabolism through different mechanisms (McCormick & Mathews, 2007).

The previous studies demonstrate that the time from weaning until adolescence is particularly sensitive to nutrition and can determine the risk of obesity in adulthood. However, this time period is also sensitive to other environmental insults that can affect

mental health throughout the lifetime (Michel et al., 2004; Boukouvalas et al., 2008). The developmental period from PD 20 to 40 in rodents is analogous to preadolescence and adolescence in humans (Spear, 2000). A surge of synaptogenesis occurs in the brain during these adolescent years. Therefore, adolescence is one of the most dynamic events of human growth and nutrition is just one example of the early life environment that has consequences on circuitry development and adult mental health. Disruption in maternal care is another example of an environmental insult that has long-lasting effects on adulthood health and behaviors. Unsatisfactory maternal care leads to dysregulation of the neuroendocrine response to stress (Plotsky & Meaney, 1993; Liu et al., 2000) and can lead to an increased risk of addiction in adulthood (Piazza & Le Moal, 1997). Because the early post natal time period is so sensitive, it may be possible to positively impact development through intervention at this time. For example, early post weaning exercise can reverse the damage done to metabolic feeding circuits caused by maternal high fat diet (Sun et al., 2013). Understanding what molecules are specifically sensitive to over nutrition during the early postnatal period will allow us to develop targets for possible positive intervention to prevent dysfunction is adulthood. Many diet induced obesity rodent studies begin the diet months after weaning (Kanoski et al., 2010; Baladi et al., 2009; Lassiter et al., 2010) and therefore results from these studies cannot be applied to childhood obesity treatments. Research is still needed to identify the periods of sensitivity in the programming of dopamine reward system in obesity and determine how permanent metabolic programming is after intervention.

The Basics of Epigenetics

The body and brain respond to a changing environment by altering gene expression patterns both acutely and on a long term basis. One reason for variations in response to a changing environment could be a gene-environment interaction. For instance, a polymorphism in an important gene could lead to increased risk of a psychiatric disorder in a stressful environment. This has been seen in epidemiological studies of serotonin receptor polymorphisms and the risk for depression (Caspi et al., 2003). However, it is also possible to have increased risk for psychiatric disorder without a corresponding change in genetic code. For example, early life stress in humans (Chapman et al., 2004) and animal models (Kaffman & Meaney, 2007) can increase the risk of depression and depressive-like behaviors. The mechanism by which early life stress is able to program the brain involves epigenetic modulation of the expression of individual genes or large clusters of related genes. Epigenetics refers to the potentially heritable, but environmentally modifiable, regulation of genetic function mediated through a mechanism that doesn't involve altering the genetic code (Russo et al, 1996; Wu and Morris, 2001). Epigenetics is viewed as the vehicle through which the environment interacts with the genome to determine function, in health and disease. Aberrations in epigenetic markers have been associated with many diseases such as cancer, diabetes and addiction (Dawson & Kouzarides, 2012; Gilbert & Liu, 2012; Feng & Nestler, 2013). DNA is wrapped around a cluster of histone proteins and covalent histone modifications, such as acetylation, phosphorylation, methylation, sumoylation and ubiquitylation, modulate gene expression via alterations in chromatin structure in conjunction with DNA methylation. These epigenetic mechanisms in total can play a role during times of development to program behavioral phenotypes in adulthood, or increase vulnerability for a psychiatric disorder caused by other phenomena.

DNA methylation has been widely studied in the context of early life environmental insults. DNA methylation occurs with the addition of a methyl group to the C5 position of cytosine (5-mC) predominantly at CpG sites (Klose and Bird, 2006). CpG islands are stretches of DNA around 1000 base pairs long that have a high CpG density (Bird *et al*, 1985). CpG islands are important for transcriptional regulation and 70% of gene promoters reside within CpG islands (Saxonov et al, 2006). The importance of CpG islands in transcription are maintained between species and conserved between mice and humans and transcription factor binding sites often found to be within and around these regions (Illingworth *et al*, 2010). Classically, DNA methylation reduces access of transcription factors to regulatory elements and causes transcriptional repression by decreasing the binding of specific transcriptional enhancers (Murgatroyd et al., 2010). DNA methylation plays a pivotal role in cell differentiation, imprinting, and X chromosome inactivation and is viewed as a more stable epigenetic mark compared to others (Martinowich et al., 2003). A family of methyl CpG-binding domain proteins, including MeCP2, and MBD1-4, bind to methylated cytosines and interact with histone deacetylases and DNA-methyltransferases to repress genes and maintain methylation (Chen et al., 2003). Methyl binding proteins, such as MeCP2, are highly expressed in the brain and necessary for proper circuitry and behavioral development (Amir et al., 1999, Skene et al., 2010).

DNA methylation is catalyzed by DNA methyltransferases (DNMTs) DNMT1, DNMT3a and DNMT3b. The expression of DNMTs normally declines in terminally differentiated cells in all tissues except for the brain. The brain is specialized for sensing the environment and responding and adapting to change. One of the mechanisms behind the ability to adapt to a changing environment is epigenetic plasticity. Both DNMT1 and DNMT3a are expressed by post mitotic neurons and serve unique functions

in the central nervous system (Goto et al., 1994). The best-studied DNMT in the nervous system is DNMT1. Unlike the other DNMTs, DNMT1 preferentially methylates hemimethylated DNA (Pradhan et al, 1999) and is involved in methylation repair and maintenance (Mortusewicz et al, 2005). Chromatin structure can be altered because of steric hindrance or charge introduced by the DNA methylation in combination with other epigenetic markers, such as histone modifications. Epigenetic mechanisms controlling transcriptional activity may be cell-type, and even gene-specific. There have been several instances discovered where DNA methylation increased transcription at some gene promoter sites (Mellen et al., 2012; Labonte et al., 2012). Changes in epigenetic markers may explain how the environment regulates the genome in specific concepts. These changes are well poised to mediate the effects of early environmental factors on adult disease and behavior.

Epigenetics and the Early Life Environment

The crucial role of the epigenetic processes in mediating maladaptive neurobiological and behavioral consequences of the early-life environment and environmental adversity has been demonstrated in animal models. Brain specific brain DNA methyltranferase knockouts have been generated and characterized and demonstrated behavioral dysfunction (Feng, et. al 2010). This was the first proof of concept that it was possible to alter behavior through epigenetic mechanisms. Further advances in the field revealed in animal models of early life stress that epigenetic mechanisms are recruited and could be the mechanism behind the observed behavioral dysfunction (McGowan et al., 2009; McGowan et al., 2010; Weaver et al., 2004). Early-life adversity triggers DNA methylation changes in several brain regions and controls

genes that mediate a depressive phenotype (Lutz et al., 2013). For example, the glucocorticoid receptor gene promoter methylation is increased by early life stress and leads to adult stress dysregulation in both human (McGowan et al., 2009) and rodent samples (Weaver et al., 2004; McGowan et al., 2010). Other studies have revealed that DNA methylation is central to various types of aversive learning (Miller et al., 2010) and synaptic plasticity (Roth et al., 2009) after early life insults. For instance DNMT1 has a role in regulating the induction of synaptic plasticity in the hippocampus (Levenson et al., 2006; Miller & Sweatt, 2007). The early life time period may provide a window where epigenetic machinery is vulnerable to insult and could change developmental trajectories and cause psychiatric dysfunction in adulthood.

Epigenetic Recruitment after Rewarding Substances

Activity-dependent methylation and demethylation of DNA is essential for the behavioral and neuronal plasticity driven not only by early life stress, but in reward-related experiences as well. Epigenetic marks have been seen to change in reward related brain regions in response to drugs of abuse (Hiroi et al., 2005). Reward learning induces changes in DNA methylation patterns that are gene specific and correlate with changes in mRNA levels (Day et al., 2013). Cocaine is one example of a drug of abuse that alters DNMT levels and DNA methylation in the NAc. The epigenetic changes are thought to program reward memory by regulating structural changes and influencing addictive behaviors such as conditioned place preference (LaPlant et al., 2010). Drug-induced neuronal plasticity is viewed as a major molecular mechanism for the development of drug addiction and relapse (Kalivas et al., 2008; Nestler, 2001; Shaham & Hope, 2005; Wong et al., 2011). Significant changes in DNA methylation have been seen after alcohol (Bonsch et al., 2004; Marutha Ravindran et al., 2004; Philibert et al., 2008), amphetamine (Deng et al., 2010), cocaine (He et al., 2006; Novikova et al.,

2008), nicotine (Philibert et al., 2009; Sata et al., 2008) and opiate (Neilsen et al., 2008) exposure in human and mouse models. These studies show that rewarding substances have the ability to recruit epigenetic machinery and cause epigenetic changes in brain circuitry that regulates behavior. Our lab identified DNA methylation as an epigenetic mechanism linking the chronic intake of HF diet with altered dopaminergic gene expression and mu opioid receptor expression (Vucetic et al., 2010; Vucetic et al., 2011). Little is known about the mechanisms that drive gene expression changes in response to diet and/or obesity. Epigenetic gene regulation, including DNA methylation, and histone modifications, represent a pathway through which organisms can rapidly adapt to nutritional challenges. There is also a strong possibility that these mechanisms are more vulnerable to alteration when the brain is still developing, such as in utero or the pre-adolescent time period. Identification of such windows will be of major public health importance, given the vast number of psychiatric and metabolic disorders linked to early life adversity and early life nutrition.

Reward System and Epigenetic Effects of Exercise on the Brain

Exercise is an example of a non-nutritional environmental exposure that recruits epigenetic machinery and causes neuroadaptations in the brain. Because it affects similar brain regions as high fat diet, it is a potential option for the prevention of neuradaptations of the reward system that occurs in obesity. Voluntary physical activity is important to human health for the prevention of obesity and it has positive effects on learning, memory, and cognition. The obesogenic environment of Western culture includes overconsumption of palatable foods and increases in sedentary lifestyle. This increase in inactivity has been associated with obesity, cardiovascular disease, Type II

Diabetes, and certain types of cancer (Powell et al., 1994). Inactivity and obesity are also associated with neurological disorders such as Alzheimer's disease and depression. There is a growing field of research that illustrates that high fat diet has a direct damaging effect on the brain. Diets high in saturated fat are known for reducing molecular substrates that support cognition and therefore increase the risk for neurological dysfunction in both humans (Greenwood et al., 2005) and animals (Molteni et al., 2002). Holloway et al. (2011) reported that healthy adults who ate a high fat diet for 5 days performed worse on tasks measuring attention and speed of retrieval than they had prior to the diet. Studies with rodents confirm chronic high fat diet produces cognitive deficits and neuroinflammation in the hippocampus (Heyward et al., 2012, McNay et al., 2010; Molteni et al., 2004 and Pistell et al., 2010). Rodents on high fat diet also have reduced levels of BDNF and CREB, which are important for neuronal plasticity (Molteni et al., 2002). Fortunately, exercise has positive actions on the brain and is a low-cost, and common way to combat obesity. Studies looking at the beneficial effects of exercise have mainly focused on learning and memory in the hippocampus. In humans, exercise counteracts the mental decline associated with aging (Kramer et al., 1999), enhances the mental capacity of young adults (Winter et al., 2007), and facilitates functional recovery after brain injury or disease (Vayman & Gomez-Pinilla, 2006).

Rodent models of voluntary wheel running have given us insight to the mechanism behind these cognitive benefits. Exercising rats show exercise promotes neurogenesis in the brain (van Praag et al., 1999) and increases expression of BDNF mRNA (Gomez-Pinilla et al., 2011). When exercise and high fat diet are paired together, exercise has the ability to reverse the damaging effects of high fat diet on synaptic plasticity in the hippocampus by normalizing BDNF levels and reducing oxidative damage (Molteni et al., 2004). Exercise can protect the hippocampus from nutritional

insults, but what about other brain regions? We now know that consumption of high fat diet also causes neuroadaptations in the mesolimbic reward system. Damage to hippocampal function by high fat diet seems to be through reduced BDNF signaling and oxidative damage. Dysfunction in the reward system after high fat diet, however, is through a different mechanism. Chronic intake of a rewarding substance leads to changes in dopamine signaling and subsequent obesity causes central resistance of metabolic hormones found in reward regions of the brain. It is possible that exercise is a way to combat the dopamine dysfunction in the mesolimbic reward system seen in obesity.

For years now, exercise has been used as a treatment for depression and other psychological disorders (Lawlor & Hopker, 2001; Blumenthal et al., 1999; Petruzzello et al., 1991). Recently, exercise has been suggested to be beneficial for treating drug addiction (Smith & Lynch, 2011). Since the mechanisms of depression, addiction, and palatable food overconsumption pathways overlap, exercise may also be beneficial for treating dopamine dysfunction in obesity. There are many theories as to why exercise is beneficial in these disorders. Exercise increases body temperature (Petruzzello et al., 1991), causes an increase in serum calcium levels (Sutoo & Akiyama, 1996) and causes an increase in monoamine synthesis (Dishman, 1997). The central belief among these researchers is that exercise acts on the same pathway of antidepressant medications in the treatment of depression. Mechanistically, physical activity and exercise activate the same reward pathway as drugs of abuse. Exercise increases dopamine concentrations and dopamine receptor binding (Greenwood et al., 2011 and MacRae et al., 1987) and may act as an alternative reward so to prevent drug administration. It is unknown how exercise in conjunction with a rewarding substance like high fat diet may interact within the reward system. Because the consumption of palatable food and administration of

addictive drugs are controlled by the dopamine reward pathway, I hypothesize that exercise can also counter the dopamine dysfunction caused by chronic high fat diet.

Exercise functions as an alternative non-drug reinforcer and can compete with the drug and reduce vulnerability to addiction. Rodents given concurrent access to a running wheel and methamphetamine self-administer less drug as compared sedentary rats (Miller et al., 2012). Similar findings have been reported for cocaine self-administration (Smith and Pitts, 2011) and was protective both when the running wheel was given concurrently or when given before drug exposure. This has possible implications for exercise as a preventive measure against dopamine dysfunction before it even begins. At the molecular level, exercise reduces drug induced dopamine release (Chen et al., 2008, Marques et al., 2008). Additionally, running increases levels of tyrosine hydroxylase, and total dopamine levels the reward pathway (Droste et al., 2006 and Greenwood et al., 2011). These neuroadaptations caused by exercise may influence an individual's vulnerability to initiate drug use and/or develop addiction. One way exercise prevents consumption of rewarding substances is that exercise has reward value of its own. In fact, rats are willing to “work,” for and show conditioned place preference to the running wheel (Belke et al., 2005; Brene et al., 2007). The reward value of exercise comes from increases in dopamine in reward regions. Microdialysis studies revealed increased in extracellular DA in the mesolimbic region after a 20-60min treadmill run that remained elevated for 2hrs (Hattori et al., 1994; Meeusen et al., 1997; Wightman et al., 2002). These findings are important because they suggest that exercise may normalize the hypofunctioning in the mesolimbic system that occurs following chronic high fat diet exposure and high fat withdrawal.

Molecules that could explain the beneficial effects of exercise in the reward system may be the same as those discovered to be beneficial in the hippocampus. For

instance, important factors in synaptic plasticity and cognitive function, such as BDNF, synapsin I, calcium/calmodulin-dependent protein kinase II (CaMKII) and cyclic AMP-responsive element (CRE)-binding protein (CREB) are enhanced in both addiction and after exercise in reward regions of the brain (Wu & Gomez-Pinilla,2007). Chronic exposure to high fat diet alters epigenetic markers that may underlie the propensity to develop neuroadaptations in reward regions. The beneficial effects of exercise may work through a similar epigenetic pathway and remodel chromatin structure in a way that prevents dopamine dysfunction (Gomez-Pinilla, 2011). Further research will be needed to see if an epigenetic pathway is activated in reward regions by exercise to prevent the dopamine dysfunction of obesity. Exercise seems to produce efficacious effects in other brain regions, therefore, researching the effects of exercise in the reward system of the obese brain will be important for future weight loss studies and the control of palatable food consumption.

Conclusion

We now see that obesity can be thought of as a disease of the central regulation of food intake and many behavioral dysfunctions and neuroadaptations are associated with the obese state. The brain is a highly adaptive organ and changes in response to multiple internal and external environmental signals. However, these changes could lead to maladaptive behavior or disease in the future. Studies described here support the finding of dopamine dysfunction in obesity patients and animals given access to high fat diet. The neuroadaptations seen after chronic high fat diet are similar to those seen after chronic intake of drugs of abuse. The following experiments will characterize how dopamine circuitry has adapted to chronic high fat diet intake at the molecular and behavioral level. Understanding whether these reward system neuroadaptations are

persistent after chronic high fat is critical for the field of obesity interventions; therefore, the following experiments also characterize the dopamine system after high fat withdrawal. We now see how critical the early nutritional environment is for optimal brain development. Early postnatal life is a dynamic period where dopamine projections are still being laid down. It is possible that the pre-adolescent period is more vulnerable to the damaging effects of high fat diet than other points in time. Overnutrition during this time period may lead to epigenetic programming and lasting neuroadaptations after intervention. The following experiments characterize chronic high fat diet in pre-adolescent male and female mice and compared them to adult animals on high fat diet. This experimental design allows us look at the effect of high fat diet on a critical time period in brain develop and assess if any permanent damage is seen in adulthood. The experimental design includes females as well as males. There are crucial sex differences in the presentation of obesity and in dopamine system activation. Bringing sex differences into the analysis of high fat intake and high fat withdrawal effects on the brain will be important contribution to the obesity intervention field. Finally, there is sufficient evidence that exercise activates the dopamine reward system and studies show exercise to be beneficial in other brain areas important for learning and memory. Exercise has the ability to be a rewarding replacement for palatable foods and may be protective against dopamine dysfunction in obesity. Access to the running wheel recruits epigenetic machinery similar to other rewarding substances and this may be the mechanism underlying its protective benefits. Obesity is a complex state and understanding the mechanisms behind obesity interventions like diet and exercise as well as how the brain changes along with increasing BMI and decreasing BMI will help us come up with viable drug targets and therapies to combat this widespread epidemic.

CHAPTER 2: Reversal of the Dopamine System Dysfunction in Response to High Fat Diet

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Introduction

Overconsumption of widely available, calorie-dense palatable food is considered a major factor contributing to the high rates of obesity in the U.S (Swinburn et al., 2009). Because palatable foods are often consumed after energy requirements have been met, the rewarding properties of palatable foods may override homeostatic satiety signals. Many neurotransmitters play a role in feeding behavior (e.g. opioids, dopamine, GABA, serotonin) as well as the integration of peripheral nutrient signals (e.g., leptin, insulin, ghrelin). Dopamine signaling is a key mediator in both food reward and reward-seeking behavior, as dopamine in the mesolimbic/ mesocortical region is associated with the

rewarding properties of food, sex, and drugs of addiction (Fibiger & Phillips, 1988). Acutely, palatable food causes a burst of dopamine in the central reward system (Hernandez et al., 1988; Sahr et al., 2008). With chronic consumption of rewarding food, the increased dopamine release over time may lead to adaptations that are associated with reward hypo-function.

Several lines of evidence support the hypothesis of altered dopamine function in obesity. Human imaging studies revealed blunted activation in reward regions of obese patients while drinking a highly palatable solution (milkshake) (Stice et al., 2008). The blunted reward response was associated with less brain dopamine receptor D2 availability. In fact, mutations in the human dopamine D2 receptor have been linked with both obesity and addiction (Noble et al., 1991). Dopamine content in the synapse is largely controlled by dopamine transporter (DAT) uptake. Dopamine transporter levels are negatively correlated with body mass index and genetic variants of DAT are also associated with obesity (Chen et al., 2008; Need et al., 2006). Animal models of obesity have demonstrated decreases in basal extracellular dopamine and reduced dopamine neurotransmission in the nucleus accumbens and ventral tegmental area (Geiger et al., 2007; Geiger et al., 2009; Cone et al., 2010). Decreases in dopamine-related genes after chronic high fat (HF) diet suggest decreased signaling in reward regions (Vucetic et al., 2012; Alsio et al., 2010; Johnson & Kenny, 2010, Huang et al., 2005). This decrease in dopamine activity after chronic high-fat diet may reduce the sensitivity to natural rewards and facilitate continued overconsumption and further weight gain.

Early life is a critical period in brain development, and the early nutritional environment can influence brain pathways controlling food intake and energy metabolism. Early exposure of mice to a high fat diet for as little as one week altered

adult caloric intake and expression of dopamine-related signaling molecules (Teegarden et al., 2009). Further, early postnatal over nutrition in mice, driven by a small litter number throughout lactation, predisposes the offspring to adulthood obesity by altering hypothalamic development (Bouret, 2010). While it is clear that early life nutrition can affect brain development and obesity risk, little is known about the relative permanence of these changes across the lifespan. Additionally, previous studies have been done in male animals but females have been rarely studied in this context. To these ends, both male and female mice were studied for changes in gene expression and dopamine metabolism after they were made obese in early life through chronic consumption of a HF diet from birth through 12 weeks of age. The dopamine system was also evaluated 4 weeks after removal of the HF diet, to examine whether the changes persisted or reversed.

Methods and Procedures

Animals and experimental model. C57BL/6J females were bred with DBA/2J males (The Jackson Laboratory, Bar Harbor, ME). All dams were fed standard control diet (#5755; 18.5% protein, 12% fat, 69.5% carbohydrate) up until parturition when half the dams/litters were placed on high fat diet (Test Diet, Richmond, IN #58G9; 18% protein, 60% fat, and 20.5% carbohydrate). Offspring were weaned at 3 weeks of age and remained on either the control diet or the high fat diet until 12 weeks of age. Body weights were recorded weekly, and both male (n=5-10) and female (n=5-10) mice were used. The Institutional Animal Care and Use Committee (IACUC) of the University of Pennsylvania approved all procedures.

Sucrose and Saccharin Preference. In separate experiments, mice were individually housed (n=8-10/group) in standard cages for 3 days with one bottle of 200 ml of the test solution (4% sucrose or 1% saccharin solution (w/v)) and another bottle with 200 ml of tap water. House chow was available *ad libitum*. Sucrose (ml), water (ml), and food consumption (g), were measured and the placement of the bottles was reversed daily. Preference was calculated using the average of the measurements from the last 2 days as follows: preference % = [(sucrose consumption/sucrose + water consumption) × 100].

Genomic DNA and Total RNA isolation from the brain. Animals (n=5/group) were euthanized with an overdose of carbon dioxide, followed by cervical dislocation; a method recommended by the Panel on Euthanasia of the American Veterinary Medical Association. Brains were then rapidly removed and placed in RNAlater (Ambion, Austin, TX) for 4-6 hours before dissection. Brain dissections to isolate the prefrontal cortex, the nucleus accumbens and the ventral tegmental area were preformed as previously described (Vucetic et al., 2010; Reyes et al., 2003; Cleck et al., 2008). Genomic DNA and total RNA were isolated simultaneously using AllPrep DNA/RNA Mini Kit (Qiagen).

Gene expression analysis by quantitative Real-Time PCR. For each individual sample, 500ng of total RNA was used in reverse transcription using High Capacity Reverse Transcription Kit (ABI, Foster City, CA). Expression of target genes was determined by quantitative RT-PCR using gene specific Taqman Probes with Taqman gene expression Master Mix (ABI) on the ABI7900HT Real-Time PCR Cycler. Gene probes are listed in supplemental material. Relative amount of each transcript was determined using delta CT values as previously described in (Pfaffl, 2001). Changes in gene expression were calculated against an unchanged GAPDH standard.

Western blot. Ventral tegmental area tissue was dissected out and flash-frozen on dry ice and stored at -80 °C. Frozen tissue was homogenized in 350 ul of 1X Extraction Buffer with 1:1000 DDT Solution and (1:1000) PIC added (Epigentek, Farmingdale, NY). Homogenates were incubated on ice for 15 minutes with vortexing every 5 minutes. Homogenates were then centrifuges at 14,000rpm for 10 minutes at 4°C. Supernatants were collected and processed for total protein concentration according to the Micro BCA procedure (Pierce, Rockford, IL, USA). Protein levels of tyrosine hydroxylase were measures by western blot analysis. Protein samples were separated by electrophoresis on a 10% polyacrylamide gel and electrotransferred to a PVDF (polyvinylidene fluoride) membrane. Non-specific binding sites were blocked in Odyssey Blocking Buffer (Licor, Lincoln, Nebraska) for 1 hr at 4°C. Membranes were incubated at 4 °C overnight, with the primary rabbit antibody anti-TH (1:200, Pel-Freez Biologics, Roger, Arkansas #p40101-0) and rinsed with 0.01% Tween20 in Tris-buffered saline. Membranes were then incubated for 1hr at room temperature with IRDye 800CW Goat anti-Rabbit IgG (H + L,), 25 µL (Licor, Lincoln, Nebraska, 1:2000 in 0.01% Tween20 Tris-buffered saline, 0.1% SDS) and washed with 0.01% Tween20 in Tris-buffered saline. Membranes were imaged with the LI-COR *Odyssey* Infrared Imaging System and TH fluorescence was normalized to GAPDH florescence.

Ex vivo Dopamine and Dopamine Metabolites. High performance liquid chromatography (HPLC) was used to measure the content of dopamine and its metabolites in the mesolimbic reward areas of the brain (n=8-12), as described previously (Mayorga et al., 2001, Vucetic et al., 2010). Brains were collected from animals and bisected into right and left hemispheres. The NAc and PFC were dissected out and quickly frozen by dry ice and stored at -80°C. The tissue was prepared for analysis by homogenization in 0.1 N perchloric acid, centrifuged at 15,000 rpm for 15

min at 2-8˚C, and the supernatant filtered. Samples were analyzed by a Bioanalytical Systems HPLC (West Lafayette, IN, USA) using a LC-4C electrochemical detector. Samples (12 ul) were injected onto a reverse phase microbore column at a flow rate of 0.6 ml/min and electrodetection set at +0.6 V. Separation for dopamine and dopamine metabolites was accomplished by a mobile phase consisting of 90-mM sodium acetate, 35-mM citric acid, 0.34-mM ethylenediamine tetraacetic acid, 1.2-mM sodium octyl sulfate, and 15% methanol v/v at a pH of 4.2. Peak heights of samples were measured and compared with standards for dopamine and its metabolite 3,4-dihydroxyphenylacetic acid (DOPAC).

Methylated DNA Immunoprecipitation (MeDIP) Assay. MeDIP assay was preformed using MagMeDIP kit (Diagenode, Denville, NJ). Methylated DNA was immunoprecipitated using 15ul of magnetic beads coated with anti-5methylcytidine antibody (Diagenode) or mouse pre-immune serum. Enrichment in MeDIP fraction was determined by quantitative RT-PCR using ChIP-qPCR Assay Master Mix (SuperArray) on the ABI7900HT Real-Time Cycler. For all genes examined, primers were obtained from SuperArray (ChIP-qPCR Assays (-01) kb tile, SuperArray) for the amplification of genomic regions spanning the CpG sites located approximately 300-500 bp upstream of the transcription start sites. MeDIP results were expressed as fold enrichment of immunoprecipitated DNA for each site. To calculate differential occupancy fold change (% enrichment), the MeDIP DNA fraction CT values were normalized to Input DNA fraction CT values.

Statistics. Gene expression analysis was performed using Student T-test comparing aged matched controls to HF and HF + recovery groups. The alpha level was adjusted for the multiple brain regions surveyed. Significance of a gene used in one

brain region was p=.05; for two regions, p=0.025, for 3 brain regions p=.016. Sucrose preference, saccharin preference, HPLC and MEDIP, body weights and corticosterone assay analyzed using one-way ANOVA to compare control, HF, and HF + recovery groups. Post-hoc Bonferonni Multiple Comparison Tests were used to compare pair-wise differences between groups. Significance for these tests was set at an alpha level of p=.05.

Results

Mice had continuous access to control diet (control) or 60% high fat diet (HFD) until 12 weeks of age. At 12 weeks of age, half of the HF-fed animals were placed on the house chow for 4 weeks (HF +recovery). In both males and females, HFD animals (circles) were heavier than controls beginning at 9 weeks of age ($p<.05$) and remained heavier than controls throughout the recovery period (Supplemental Figure 1).

Sucrose and saccharin preference tests were administered to assess animals' response to natural and nonnutritive rewarding stimuli. Sucrose preference but not saccharin preference was altered after HF diet exposure and returned to normal levels after HFD recovery in males and females. One-way ANOVA revealed sucrose preference was significantly decreased in males (Fig. 1A) and trended toward a decrease in females (Fig. 1B) after HFD exposure ($F(2,16)=4.82$, $p<.05$; $F(2,16)=5.41$, $p<.06$, respectively). After removal of the HFD, this behavior normalized and sucrose preference no longer differed from controls. Saccharin preference was not altered in either males (Fig. 1C) or females (Fig. 1D) as a result of HFD exposure.

Because dopamine is a key regulator of reward behavior, dopamine-related gene expression was examined within the reward circuitry of a separate cohort of males and females after 12 weeks on the HFD, and in an additional group, after 4 weeks recovery from the HFD. Table 1 summarizes the gene expression patterns and statistical analysis in the VTA, PFC and NAc. In the VTA, three genes important in regulating dopamine levels at synaptic terminals were measured: catecholamine methyl transferase (COMT) involved in inactivation of catecholamine neurotransmitters; dopamine transporter (DAT), membrane spanning pump that clears dopamine from the synapse, and tyrosine hydroxylase (TH), the rate-limiting enzyme for dopamine synthesis. Fold change values for each group were determined using aged matched controls (e.g. both control time points are set to 1, and for clarity only the control for HFD is depicted on the graph). Student's t-test (n=5/group) revealed in the male VTA that COMT, DAT, and TH mRNA were significantly decreased by HFD exposure (Fig 2A) and returned to or exceeded the control levels after a recovery period off the diet (HF +recovery).

In the PFC and NAC, genes important for dopamine signaling and dopamine turnover were examined (n=5/group): COMT; protein phosphatase 1 regulatory subunit 1B (DARPP-32), a down stream signaling protein regulated by receptor stimulation; dopamine receptor D1 (DRD1), a postsynaptic G-protein coupled receptor that stimulates adenylyl cyclase; and dopamine receptor D2 (DRD2), a postsynaptic G-protein coupled receptor that inhibits adenylyl cyclase. In the male PFC (Fig. 2B), DARPP-32 was increased, while DRD1, and DRD2 were decreased after HFD exposure, and these changes persisted after the removal of the HFD (although the increase in DARPP-32 mRNA was not statistically reliable). In the male NAC (Fig. 2C), COMT, DRD1, and DRD2 were decreased by HFD exposure, and remained below

control levels after removal of the HFD. DARPP-32 levels were increased by HFD, but significantly decreased from controls after 4 weeks off the HFD.

The same brain regions and genes were examined in female mice (n=5/group). As shown in Table 2, there were significant differences observed in the pattern of gene expression in response to the HFD, as well as to the recovery off the diet. Similar to males, in the VTA, mRNA levels of COMT, and TH were significantly decreased after HFD exposure (Fig 2D). However, unlike the males, these changes persisted after the removal of the HFD. Further, in direct opposition to the pattern observed in males, HFD exposure increased DAT mRNA expression in the VTA in females, and after removal of the HFD levels were even lower than age matched controls. In the PFC, only DARPP-32 was affected by chronic HFD, with a significant increase in mRNA levels after 12 week HFD, and a return to control levels after removal of the HFD. Both COMT and D1R mRNA were significantly decreased after 4 weeks off the HFD. In the female NAC, COMT, DRD1, and DRD2 were all decreased after HFD exposure (Fig. 2F). DRD1 and DRD2 recovered to control levels after diet removal, while COMT remained levels remained significantly decreased after 4wk recovery. Protein levels of TH were quantified in the ventral tegmental area to confirm gene expression results. Chronic high fat diet was decreased in males (Figure 3A $F(2,18)=3.502$, $p=.0519$) and remained decreased after 4 week recovery period, however post hoc analysis showed this decrease was only a trend not statistically reliable. Protein levels of TH in the VTA of females (Figure 3B $F(2,19)=3.544$, $p=0.0492$) were also decreased after high fat diet and levels trended toward normalization after a 4 week recovery period.

Given the consistent decrease in gene expression for dopamine regulating genes in the VTA, dopamine and dopamine metabolites were quantified in regions that receive

projections from the VTA, the PFC and NAC. Figure 3 shows dopamine (DA) and the dopamine metabolite (DOPAC) from the PFC and NAC in males (Fig. 4A, 3C) and females (Fig. 4B, 3D). In males, exposure to the HFD produced a decrease in dopamine levels in PFC (Fig. 4A) and NAC (Fig 4C) ($F(2,13)=3.95$;$F(2,18)=3.536$, $p<.05$), which recovered after HFD removal only in the NAC. Dopamine turnover (DOPAC:DA ratio) increased in male PFC ($F(2,12)=3.85$, $p<.05$) and NAC ($F(2,17)=4.69$, $p<.05$). In contrast, the effect of HFD on DA and DOPAC in females was qualitatively different than in males. In the PFC, HFD did not affect DA or DOPAC levels. In the NAc, DA levels were decreased in HFD-fed animals and remained decreased even after removal of the HFD (Fig. 4D, $F(2,23)=4.79$, $p<.05$). DOPAC levels were unchanged in the NAc of females, which resulted in an increase in DA turnover (DOPAC:DA ratio) ($F(2,23)=7.00$, $p<.01$).

Given that DAT transcription can be regulated by differential DNA methylation and the observation of a notable sex difference in the expression of DAT in the VTA, DNA methylation in the promoter region of DAT was examined. In Figure 5A, 5C DAT gene expression in the VTA is presented again for clarity (taken from Fig 2A and 2D). DAT promoter methylation was significantly increased in males (Fig. 5B) after HFD and returned to control levels in HFD + recovery males ($F(2,11)=23.64$, $p<.01$). In females, DAT promoter methylation trended toward decrease in HFD animals (D) and was significantly decreased in HFD + recovery females (Fig 5D, $F(2,12)=5.70$, $p<.05$).

To assess if removal of the HFD in the recovery period was a stressor, baseline plasma corticosterone levels (ug/dl) were taken in control, HFD exposed (12 weeks), HFD +1wk recovery and HFD + 4wk recovery groups (n=5/group, Supplemental Fig. 2).

One-way ANOVA revealed no significant differences between groups in male animals ($F(3,16)=3.21$, n.s.).

Discussion

Chronic consumption of a high fat diet (HFD) beginning in early life was used to establish diet-induced obesity in mice. Mice displayed decreased sucrose preference and evidence of reduced dopaminergic tone in reward regions of the brain. After 4 weeks off the HFD, sucrose preference normalized in both males and females, however, some dopamine gene expression changes persisted. These experiments provide important new data describing the effect of chronic HFD on the brain reward system, highlighting the capacity for recovery and key sex differences between male and female mice.

In the HFD fed animals, a decreased sucrose preference was observed, which reversed after a recovery period. These findings extend our previous report of HFD intake driving a reduced sucrose preference (Vucetic et al., 2003) by demonstrating that this can occur with a shorter duration of HFD exposure (12 weeks versus 22 weeks), and importantly, that the response recovers in the absence of HFD. Female mice demonstrated the same response patterns as males. These findings are consistent with others in the literature which have shown through the inclusion of a pair fed group that chronic HFD, and not obesity per se, attenuates the response for sucrose in an operant task (Davis et al., 2008). Similarly, in the current study, sucrose preference recovered after 4 weeks off the HFD, while body weight remained significantly elevated, supporting

the conclusion that decreased sucrose preference was driven by the HFD exposure and not the accompanying body weight gain. It was particularly interesting that there was no change in saccharin preference. This may indicate that chronic HFD differentially affects the response to caloric and non-caloric sweet rewards. Post ingestive effects have been shown to influence preference independent of palatability, as sucrose intake has been shown to induce dopamine release in "sweet-blind" taste receptor knockout mice (de Araujo et al., 2008), nutritional value is required for reward and reinforcement (Beeler et al., 2012) and taste-independent metabolic sensing pathways have been defined in drosophila (Dus et al., 2011). Saccharin is significantly sweeter than sucrose, so an effort was made to establish equivalency in sweetness (typically 4-10x higher concentration of sucrose (Beeler et al., 2012)) however the overall preference for saccharin was lower than that for sucrose in these animals. Therefore, an alternative explanation may be that HFD differentially affected the sucrose preference because it was relatively more rewarding than saccharin (high vs. low value reward), although animals still evinced a strong preference for saccharin (~75-80% preference for saccharin compared to ~85-90% preference for sucrose).

Overall, dopaminergic gene expression within the VTA, NAc and PFC was decreased in male mice following chronic HFD. These findings are consistent with other studies that observed decreases in dopamine related genes in response to HFD (Vucetic et al., 2012; Alsio et al., 2010; Huang et al., 2005). Decreases in dopamine D2 receptor expression and function have been observed in human imaging studies (Stice et al., 2008; Wang et al., 2001) and rodent obesity models (Johnson & Kenny, 2010; Huang et al., 2006). Decreased dopamine signaling reduces the sensitivity to natural rewards and may therefore facilitate continued overconsumption of palatable foods and further weight gain (Fortuna et al., 2012; Koob et al., 2008). Further, disrupted dopamine homeostasis

driven through decreased DAT surface expression is known to drive increased intake of high fat diet (Speed et al., 2008). An exception to this pattern was seen with DARPP-32, a dopamine- and cyclic AMP-regulated phosphoprotein, which increased after HFD in NAc and PFC. DARPP-32 plays a key role in integrating a variety of biochemical and behavioral responses controlled by dopamine. It may be that DARPP-32 upregulation was compensatory in response to the chronic down regulation of D1R. In a similar model (12 wk HFD in mice), it has been shown that D1R down regulation was matched by an increase in phosphorylation of DARPP-32 in NAc (Sharma et al., 2012).

Few studies have examined the capacity for recovery of these changes after removal of the HFD. However, in two recent reports, gene expression changes and reward system defects persisted after a short withdrawal period (14-18d) (Alsio et al., 2010; Johnson & Kenny, 2010). In contrast, studies in obese patients before and after gastric bypass surgery have shown a reversal of dopaminergic changes after a longer period of weight loss (Steele et al., 2009). In males, the pattern of recovery varied by brain region. In the VTA, the observed decreases in COMT, DAT, and TH were all normalized with the removal of the HFD. In contrast, all gene expression changes observed in the NAc and PFC did not normalize. In the current study, chronic HFD led to significant weight gain and after 4 weeks off the diet, animals were still significantly heavier than controls. Therefore, the subsequent metabolic and hormonal changes that accompany obesity (e.g., increased leptin, elevated adipokines) were likely still present at 4 weeks off the diet. Therefore, gene expression changes that normalized (e.g., in the VTA) may have been primarily driven by the HFD, while those that were maintained (in NAc and PFC) may be more tightly coupled to obesity. Maintenance of weight loss by dieting is characteristically low (with 67% (Phelan et al., 2010) to 80% (Field et al., 2001) of patients regaining the lost weight). This persistence of gene expression changes in

reward regions could be important in partly explaining this common occurrence. It is also important to note that the observed behavioral and gene expression changes are not likely to be due to stress associated with changing diets, as there were no significant changes in basal plasma corticosterone levels on the HFD or after 1wk or 4wk recovery.

Interesting sex differences were revealed, in both the response to chronic HFD, as well as in response to diet removal. Females were similar to males in showing an overall decrease in dopamine related genes that would predict a decrease in DA activity, particularly in the VTA and the NAc. One noteworthy sex difference was the increase in DAT mRNA expression in the female VTA after HFD. This difference in gene expression, coupled with similar decreases in TH gene expression in both sexes, would suggest significant differences in dopamine neurotransmission within the NAc, both at the end of the HFD exposure as well as after the recovery period. A greater appreciation for the functional significance of these differences is an important focus of future research.

Additionally, while COMT and TH decreases recovered in the male VTA, these decreases persisted in the females after 4-week off the HFD. It is yet to be determined whether these differences would reverse with a longer time off the diet, however, it supports the conclusion that females are, at the very least slower to recover, if they recover at all. Protein levels of TH matched gene expression levels on the high fat diet but were not significantly decreased after a 4 week recovery period in both males and females. Further, gene expression changes of D1R and D2R in NAc and PFC were quite different between males and females. In males, there was a general decrease in gene expression in both regions that largely persisted after diet removal. In females, D1R and D2R were decreased in the NAc and then recovered, but there was no effect of HFD on dopamine receptors in PFC. In the current studies, female animals were sacrificed

without accounting for estrus stage. While some of the observed endpoints are known to vary across the estrus cycle, female animals in this study did not demonstrate increased variance across the endpoints, particularly when compared to the effect of the diet manipulations.

To complement the gene expression and protein findings, dopamine was measured in the primary projection regions of the VTA, namely the PFC and the NAc. Dopamine levels tended to parallel changes seen in TH mRNA in the VTA. In the NAc of both males and females, levels of DA decreased in response to the HFD diet; a response which recovered in males, but not females. In the PFC, dopamine levels were also decreased by HFD, however, there was no recovery off the diet in the PFC. Additionally, females had lower levels of dopamine in the prefrontal cortex than males. Sex differences in DAT expression and function are well known in the literature, with females demonstrating increased DAT expression (Morissette et al., 1993) and function (Bhatt et al., 2005), and these differences may contribute to the different baseline levels of dopamine between males and females. Examination of the DOPAC:DA ratio is informative as well. An increase in this ratio may have reflected a compensatory response driven by decreases in DA. The long-term functional significance of these changes in dopamine metabolism would be illuminated by measuring changes in dopamine release using *in vivo* microdialysis.

Moreover, these data identify dynamic regulation of DNA methylation within the promoter of the DAT gene, particularly in the males. Recently, we have demonstrated that DAT expression can be dynamically regulated by differential DNA methylation in response to HFD (Vucetic et al., 2012), and that increased DAT promoter methylation correlates with a decrease in gene expression. Here we identify the plasticity of this

response, as the increased DNA methylation (and decreased expression of the mRNA) seen in males reverses upon removal of the HFD. Epigenetic gene regulation, for example through changes in DNA methylation, presents a pathway whereby organisms can readily adapt to environmental challenges. Epigenetic marks can be maintained across the lifespan (Ollikainen et al., 2010), and in cultured embryonic stem cells, both reversible and persistent patterns of differential DNA methylation were observed in response to changing environmental conditions (Tompkins et al., 2012). These data are the first to demonstrate *in vivo* a dynamic methylation pattern that changes with the presence or absence of an environmental challenge. It was notable that this same pattern was not observed in females. While the initial response to the HFD was as predicted (decreased DNA methylation driving increased gene expression), this pattern was not maintained throughout the recovery period. This suggests that DNA methylation and gene expression may become uncoupled during the four weeks off the HFD or it may suggest that DAT mRNA is regulated by other means in females.

In males, sucrose preference, DA-related gene expression in the VTA, and dopamine in the NAc follow a consistent pattern, of suppression in response to the chronic HFD that recovers after removal of the diet. Interestingly, while the behavioral responses to sucrose are similar in the females, both the gene expression pattern and NAc dopamine levels show a lack of recovery upon removal of the HFD. Reward-related behaviors are clearly influenced by additional neurotransmitter systems such as the opioids, and perhaps in females, the behavioral response to sucrose is more strongly associated with changes in opioids. Overall, the present data suggest that sex differences in the both the initial response to HFD, as well as to recovery after removal of the HFD, with regard to dopamine-related gene expression represent an important direction for future research directed at how chronic consumption of a HFD impacts the

brain reward system. Most notably, these data identify significant plasticity in the dopaminergic response to HFD, suggesting that while the adverse effects of chronic HFD consumption and/or obesity are significant, the potential for recovery exists.

Table and Figure Legends

Table 1. Gene Expression Summary and Statistics in Males. Gene expression results for male ventral tegmental area (VTA), prefrontal cortex (PFC), and nucleus accumbens. Summary of fold change, significance and p values are presented for HF and HF +recovery groups as compared to their age matched control.

Table 2. Gene Expression Summary and Statistics in Females. Gene expression results for male ventral tegmental area (VTA), prefrontal cortex (PFC), and nucleus accumbens. Summary of fold change, significance and p values are presented for HF and HF +recovery groups as compared to their age matched control.

Figure 1. Sucrose preference but not saccharin preference is altered after high-fat diet (HFD) exposure and returns to control levels after HFD recovery in males and females. Sucrose preference (A,B) and saccharin preference (C,D) were evaluated in counter balanced order of control (white bars) HFD (black bars) and HFD + recovery (patterned bars) males and females. (n=8/group) *p<.05 significantly different from control group.

Figure 2. Chronic high-fat diet (HFD) and recovery after HFD alters dopamine related gene expression in males and females. Gene expression was measured in the ventral tegmental area (VTA; A,D.), prefrontal cortex (PFC; B,E), and nucleus accumbens (NAc; C,F) of control (white bars), HFD (black bars) and HFD + recovery

(patterned bars) males and female mice (n=5/group). HFD and HF + recovery groups are fold change from their own aged matched controls. Only one control group (12wk) is shown and set to 1. *p<.05, **p<.01, ***p<.001, ****p<.0001 significantly different from own control group.

Figure 3. Chronic high fat diet (HFD) reduced TH protein levels in the ventral tegmental area in both males and females. Protein levels of TH were visualized in the VTA of both males (A) and females (B) and representative western blots are shown. C) Quantification of TH protein florescence normalized to GAPDH from all male samples (n=8). D) Quantification of TH protein florescence normalized to GAPDH from all female samples (n=9). Statistically significant results are shown at an alpha level of p=0.05

Figure 4. Decrease in Dopamine levels in PFC and NAC after HFD from birth and mixed recovery after HFD removal. Dopamine and dopamine metabolites were measured in the PFC (A,B) and NAC (C,D) of control (white bars), HF from birth (black bars) and HF + recovery (patterned bars). (n=8-10/group) *p<.05, **p<.01 significantly different from control group.

Figure 5. Changes in DNA methylation status of DAT promoter parallel changes in gene expression in the VTA. Genomic DNA was isolated from the dissected VTA of control (white bars), HF from birth (black bars) and HF + recovery (patterned bars). DNA was digested by restriction enzyme MSE1 and immunoprecipitated by a 5-methylcytosine antibody. The enrichment of DNA methylation relative to input DNA in the promoter region of DAT was quantified by qPCR (B, D). DAT mRNA levels, (A, C; previously presented in Fig 1) are shown for comparison (n=5/group). *p<.05, **p<.01, ***p<.001 significantly different from control group.

Supplementary Information Description

Supplemental Figure 1. Body weights increase after 12 weeks HF diet and do not normalize after 4wk recovery period. HF from birth animals (black circles) were placed on 60% HF diet at postnatal day 1. After 12 weeks HF diet exposure, half the animals were placed on control diet for a recovery period. Red line depicts time on HFD. Control animals (black squares) remained on standard chow ad libitum throughout the experiment. (n=5/time point/group) *p<.05, **p<.01, ***p<.001, ****p<.0001 significantly different from control group.

Supplemental Figure 2. Corticosterone levels in males were not different after 12 weeks HF diet or after HF + recovery. Plasma corticosterone levels (ug/dl) were taken from trunk blood in control (white bar), HF from birth (black bar), HF +1wk recovery (gray bar), and HF +4wk recovery (striped bar). Blood was taken at same time of day during the light phase.

Taqman Gene Expression Primers:

COMT Mm00514377_m1

DAT Mm00438388_m1

TH Mm00447557_m1*

DARPP-32 Mm00454892_m1

DRD1 Mm01353211_m1

DRD2 Mm00438545_m1

Epitect ChIP qPCR Assay for MedIP assay:

DAT Slc6a3, nM_010020.3(-)01Kb

Tables and Figures

Table 1. Gene Expression Summary and Statistics in Males.

MALES				
Gene	**12wk HFD Fold change**	**P value**	**4wk Recovery Fold Change**	**P value**
VTA				
COMT	0.360	<0.001	1.93	<0.01
DAT	0.423	<0.001	1.46	<0.05
TH	0.380	<0.01	1.15	0.145
PFC				
COMT	0.748	0.0662	.586	<0.01
DARPP-32	1.62	<0.01	1.55	0.0386
DRD1	0.373	<0.001	0.553	<0.01
DRD2	0.522	<0.01	0.450	<0.01
NAC				
COMT	0.402	<0.01	0.133	<0.0001
DARPP-32	2.03	<0.025	0.235	<0.001
DRD1	0.521	<0.01	0.189	<0.01
DRD2	0.512	<0.001	0.190	<0.01

Table 2. Gene Expression Summary and Statistics in Females

FEMALES				
Gene	**12wk HFD Fold change**	**P value**	**4wk Recovery Fold Change**	**P value**
VTA				
COMT	0.502	<0.0001	0.074	<0.0001
DAT	1.93	<0.0001	0.171	<0.0001
TH	0.648	<.05	0.270	<0.0001
PFC				
COMT	0.776	<0.025	0.376	<0.0001
DARPP-32	1.98	<0.001	0.853	0.213
DRD1	1.21	0.397	0.567	<0.025
DRD2	1.14	0.662	0.670	0.1566
NAC				
COMT	0.678	<0.01	0.171	<0.0001
DARPP-32	0.830	0.292	2.05	0.0521
DRD1	0.476	<0.0001	0.767	0.186
DRD2	0.489	<0.0001	0.749	0.0631

Figure 1.

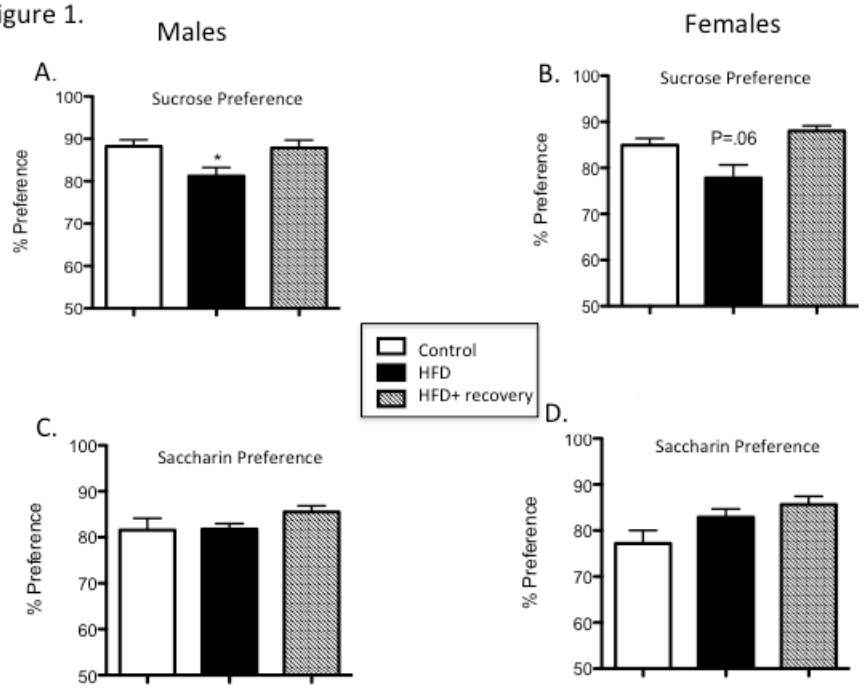

Males
Females
A.
Sucrose Preference
% Preference
100
90
80
70
60
50
*
B.
Sucrose Preference
P=.06
Control
HFD
HFD+ recovery
C.
Saccharin Preference
D.
Saccharin Preference

Figure 2.

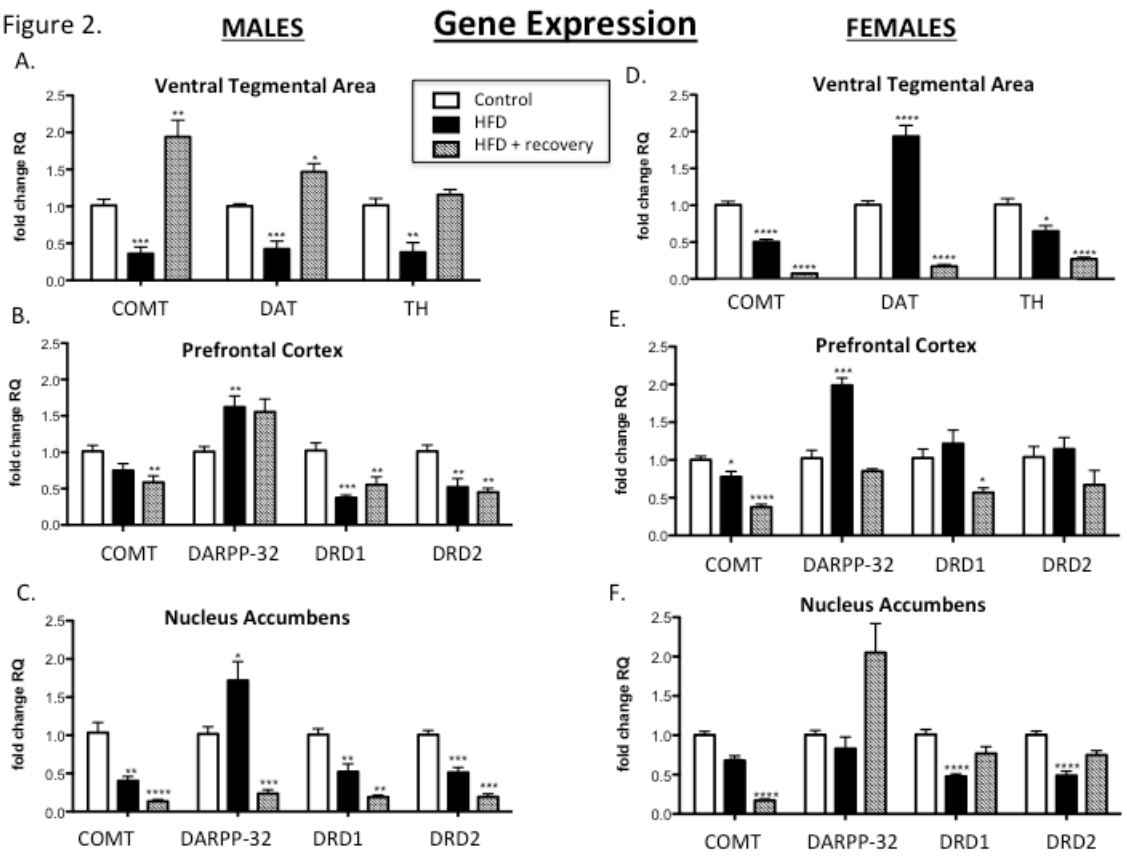

MALES
Gene Expression
FEMALES
A.
Ventral Tegmental Area
Control
HFD
HFD + recovery
fold change RQ
COMT
DAT
TH
D.
Ventral Tegmental Area
B.
Prefrontal Cortex
COMT
DARPP-32
DRD1
DRD2
E.
Prefrontal Cortex
C.
Nucleus Accumbens
F.
Nucleus Accumbens

Figure 3. Tyrosine Hydroxylase Protein Levels

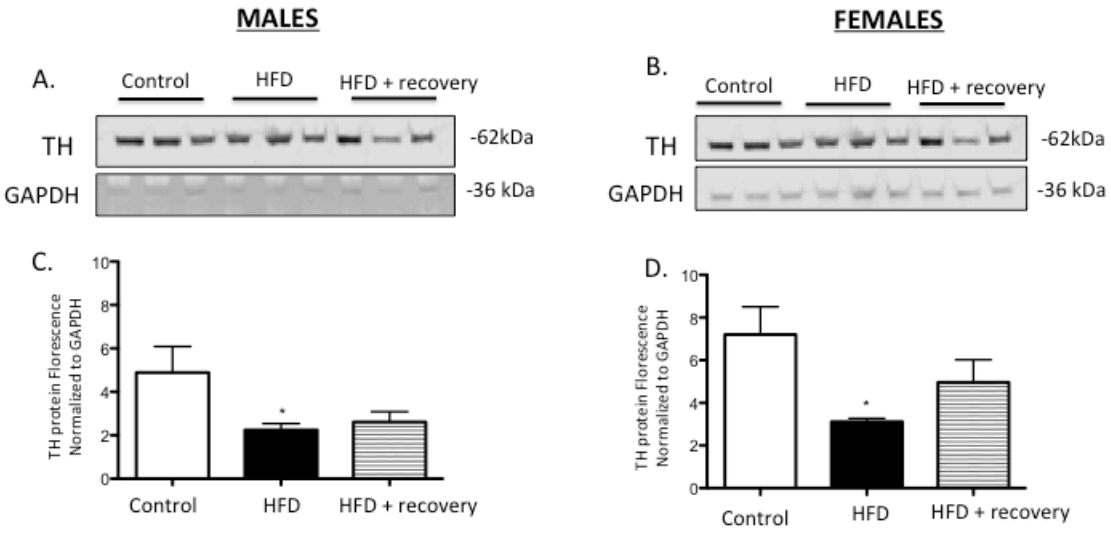


Figure 4.

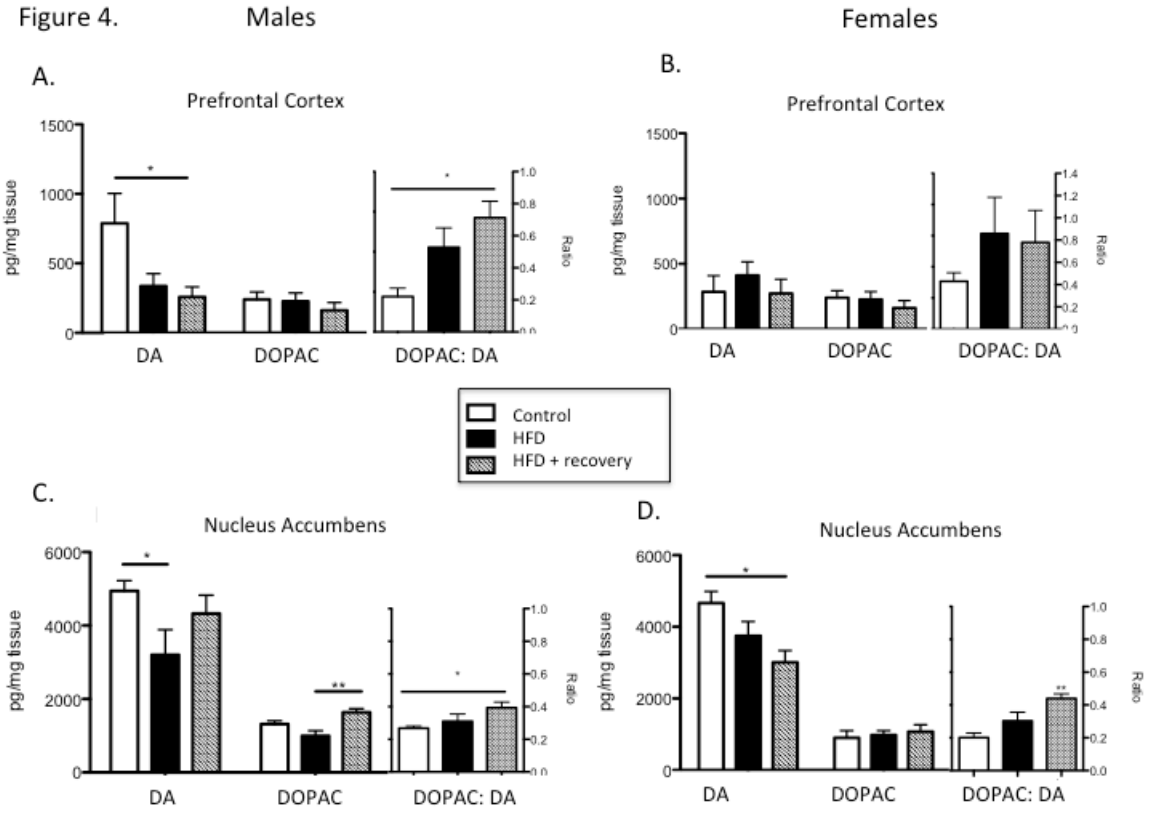
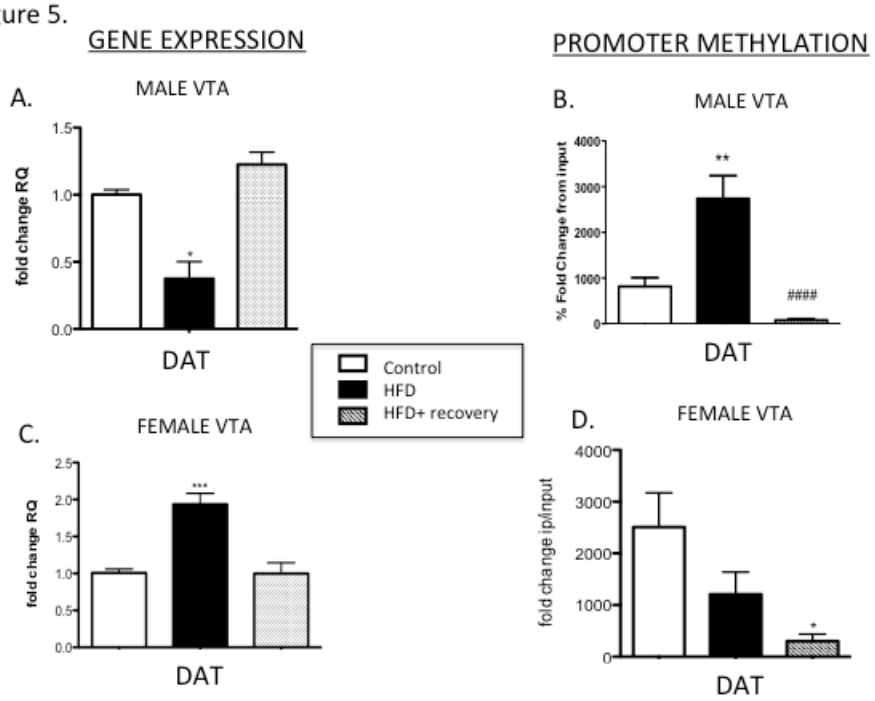
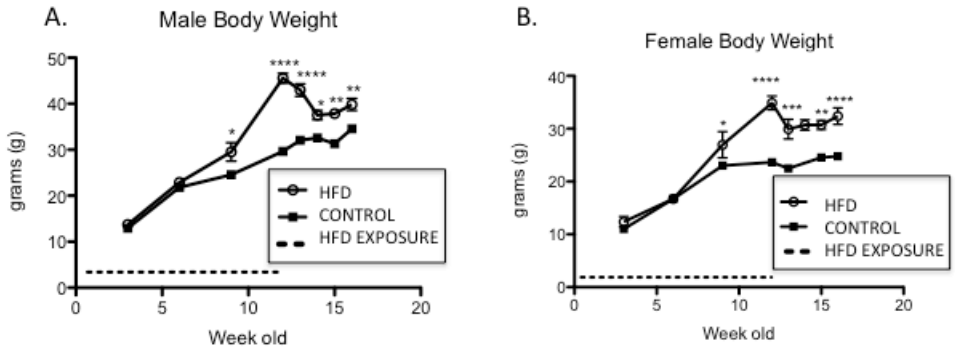

Males
Females
A.
Prefrontal Cortex
pg/mg tissue
Ratio
DA
DOPAC
DOPAC: DA
B.
Prefrontal Cortex
Control
HFD
HFD + recovery
C.
Nucleus Accumbens
D.
Nucleus Accumbens

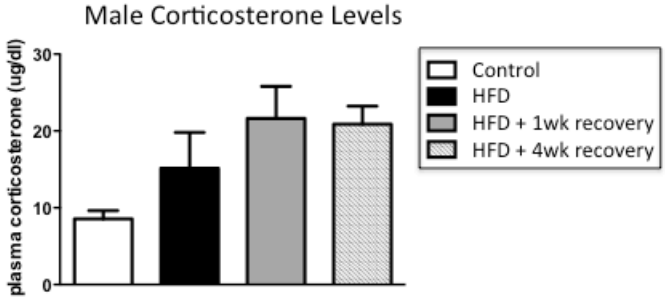
Figure 5.


GENE EXPRESSION
PROMOTER METHYLATION
A.
MALE VTA
fold change RQ
1.5
1.0
0.5
0.0
*
DAT
B.
MALE VTA
% Fold Change from input
4000
3000
2000
1000
0
**
####
DAT
Control
HFD
HFD+ recovery
C.
FEMALE VTA
fold change RQ
2.5
2.0
1.5
1.0
0.5
0.0
***
DAT
D.
FEMALE VTA
fold change ip/input
4000
3000
2000
1000
0
*
DAT

Supplemental Fig. 1



Supplemental Fig. 2



CHAPTER 3: Response of the Dopamine Reward System to High Fat Diet and Diet Withdrawal Depends on Age of Onset and Sex

Jesse L. Carlin, Sarah Mckee, Tiffany-Hill Smith, Nicola Grissom, Robert George, Irwin Lucki, Teresa M Reyes

Introduction

Obesity rates have risen at an alarming rate in both children and adults over the last decade (WHO, 2012). Obese children are more likely become obese adults with all associated health issues (Reilly & Kelly, 2011; Wija et al., 2010; Wake M., et al., 2010) and find it more difficult to lose weight in the future (Cockrell Skinner et al., 2013). Overconsumption of readily available palatable food is one factor driving the increased incidence of obesity in the U.S. Palatable foods are rewarding and acute consumption releases the neurotransmitter dopamine, in a manner similar to the response to drugs of abuse (Gieger et al., 2009). Repeated stimulation of dopamine (DA) reward pathways is thought to cause adaptations in neurotransmitter levels and brain circuitry. These adaptations can lead to an increase in compulsive behaviors and over consumption of food/drugs of abuse (Volkow & Li, 2004; Verbeken et al., 2012). There is evidence of dopamine dysregulation in obese patients. Obese individuals have decreased dopamine receptor D2 binding (Wang et al., 2001) and decreased caudate activity (Stice et al., 2008). Human dopamine D2 receptor mutations have previously been linked with compulsive disorders and drug addiction (Blum et al., 1991; Vereczkei et al., 2013, Wang et al, 2013). Palatable foods are often consumed when energy requirement have already been met, indicating dysfunction in food intake behaviors. In fact, imaging studies have revealed obese individuals have decreased activation in reward regions in response to palatable food consumption (Stice et al., 201114,15; DelParigi et al., 2004).

The neuroadaptations that occur after chronic high fat intake alter the response to drugs that act on the dopamine reward system; however, the reported effects have been mixed. Some studies have shown the high fat diet to have a dampening effect on

the rewarding aspects of drugs. Rodents on a high fat diet (HFD) have exhibited reduced cocaine self administration (Wellman et al., 2007) and reduced conditioned place preference for amphetamines (Davis et al., 2008). Rodents prone to develop obesity show a reduced conditioned place preference for cocaine (Thanos et al., 2010). Johnson & Kenny (2010) have reported reward dysfunction in obesity after rodents given high fat diet had increased brain stimulation reward thresholds. One possible mechanism behind the decrease reward value could be a decrease in basal extracellular DA (Gieger et al., 2009) and reduced DA reuptake in the nucleus accumbens (NAc) (Cone et al., 2012) in obese animals. Reward dysfunction has also been associated with decreases in dopamine-related gene expression (Carlin et al., 2013; Alsio et al., 2010, Huang et al., 2005). On the other hand, other studies have shown high fat diet to have a stimulating effect on drugs acting in the dopamine reward system. Baladi et al. (2011) reported an increase in cocaine-induced locomotion after high fat diet intake in adolescent and adult rats. Additionally, a separate study reported obesity-prone rodents to have increased extracellular dopamine (Narayanaswami, et al., 2013). These results demonstrate that high fat diet alters the dopamine reward system, but direction and scale of effect may depend on factors such as diet composition, length of exposure and age of the animals. In fact, there are significant developmental and sex-related differences found in dopamine neurochemistry (Rivest et al. 1999; Walker et al. 2000). Understanding the critical factors that regulate the neuroadaptations seen in obesity may help the treatment of the disease and the associated overconsumption of palatable foods.

Dopamine transporter (DAT) is a membrane-spanning pump that regulates synaptic dopamine concentration by reuptake of the neurotransmitter into the synaptic terminals and could be the target regulating the age and sex differences seen after high fat diet. In obese individuals, DAT levels are reduced and negatively correlate with body

mass index (Chen et al., 2008). Disruption of DAT expression increases synaptic dopamine, elevates food intake, and increases preference for palatable foods (Pecina, et al., 2003). Changes in dopamine levels in the synapse could alter the sensitivity to natural rewards and facilitate even more overconsumption and further weight gain (Johnson & Kenny, 2010; Pecina et al, 2003; McCutcheon et al., 2012).

Few studies have looked at the dopamine system after removal of the high fat diet (Johnson & Kenny, 2010; Alsio et al., 2010, Steele et al., 2009) and none of these studies have taken into account the age of onset of high-fat exposure. Research models have also shown the importance of the early life environments in affecting behavioral outcomes in adulthood (Zhu et al., 2010; Carlin et al., 2013b; Weaver et al., 2004; Murgatroyd et al., 2009; Schwarz et al., 2011;). The first postnatal day through adolescence is a particularly sensitive time period for brain development. Like early life exposure to drugs of abuse, early exposure to HFD may induce stable changes in circuitry that remain stable even after insult is removed. It has been increasingly understood that timing of HFD modulates the development of adulthood reward seeking behaviors (Teegarden et al., 2009; Labouesse et al., 2013; Naef et al., 2013; Naef et al., 2012). The following experiments were designed to test the hypothesis that beginning a high fat diet earlier in life would result in greater and more lasting changes in the dopamine reward system. Further, they look to determine if age and sex are factors in the development and persistence of dopamine changes and overconsumption of palatable foods seen in obesity. Behavioral tests were preformed by the Gomez-Pinilla lab at UCLA and the neurochemistry and gene expression experiments were performed by the Reyes Lab at UPenn.

Materials and Methods

Animals and experimental model. C57BL/6J females were bred with DBA/2J males (The Jackson Laboratory, Bar Harbor, ME) and were fed standard control chow (#5755; 18.5% protein, 12% fat, 69.5% carbohydrate) throughout pregnancy and lactation. Offspring were weaned at 3wks of and a third of animals were then placed on high fat diet (Test Diet, Richmond, IN #58G9; 18% protein, 60% fat, and 20.5% carbohydrate). Another third of animals were placed on high fat diet at 6wks of age. The final third were kept on standard hour chow. Animals remained on high fat diet for 12 weeks. A separate cohort after 12 weeks ad lib access to high fat diet was then switched to control chow. Body weights were recorded weekly, and both male (n=5-10/experiment) and female (n=5-10/experiment) mice were used. The Institutional Animal Care and Use Committee (IACUC) of the University of Pennsylvania approved all procedures.

Fat pad Weights. Animals (n=5-10/group) were euthanized with an overdose of carbon dioxide, followed by cervical dislocation; a method recommended by the Panel on Euthanasia of the American Veterinary Medical Association. Body weights were taken and white adipose tissue was removed. Specifically, the abdominal fat pads, the gonadal fat pads, and the inguinal fat pads were weighed separately. Normalization of fat pads to body weight was calculated.

Sucrose Preference. Mice were individually housed in standard cages for 3 days with access to ad lib food and two bottle choice. One bottle was filled with 200 ml of 4% sucrose solution (w/v), another with 200 ml of tap water. Sucrose (ml), water (ml), and food consumption (g), were measured. Preference was calculated using the

measurements from the last 2 days only as follows: preference % = [(sucrose consumption/sucrose + water consumption) × 100]. (n=8-10/group)

One-Hour Palatable Food Intake. Mice were individually housed (n=11-12/group) in standard cages for a total of 8 days. Each day, home cage food and body weight were measured. Animals were given access to a bowl with palatable high fat/high sugar food (Peanut Butter Chips) or control diet for one hour and measured. Daily caloric intake of ad libitum chow, palatable food, and percent calories/day were calculated. Because presence of high fat diet could have an effect on intake of palatable food, a separate cohort of Pre-Adolescent HF and Adult HF were acutely placed on ad libitum control chow during the experiment. This group is labeled HF+1wk recovery and compared to HF and HF + 4wk recovery groups.

Genomic DNA and Total RNA isolation from the brain. Animals (n=5/group) were euthanized with an overdose of carbon dioxide, followed by cervical dislocation; a method recommended by the Panel on Euthanasia of the American Veterinary Medical Association. Brains were then rapidly removed and placed in RNAlater (Ambion, Austin, TX) for 4-6 hours before dissection. Brain dissections to isolate the prefrontal cortex, the nucleus accumbens and the ventral tegmental area were preformed as previously described (Vucetic et al. 2010, Reyes et al. 2003). Genomic DNA and total RNA were isolated simultaneously using AllPrep DNA/RNA Mini Kit (Qiagen).

Gene expression analysis by quantitative Real-Time PCR. For each individual sample, 500ng of total RNA was used in reverse transcription using High Capacity Reverse Transcription Kit (ABI, Foster City, CA). Expression of target genes was determined by quantitative RT-PCR using gene specific Taqman Probes with Taqman gene expression Master Mix (ABI) on the ABI7900HT Real-Time PCR Cycler.

Gene probes are listed in supplemental material. Relative amount of each transcript was determined using delta CT values as previously described in (Pfaffl 2001). Changes in gene expression were calculated against an unchanged GAPDH standard.

Ex vivo Dopamine and Dopamine Metabolite Measurement by HPLC. High performance liquid chromatography will be used to measure dopamine content of the mesolimbic reward areas of the brain (n=8-12). Brains will be collected from animals and cut into right and left hemispheres. NAc and PFC will be dissected out and quickly frozen by dry ice and stored at -80°C. Samples will be analyzed by the Bioanalytical Systems HPLC (West Lafayette, IN, USA) with a PM-80 pump, a Sample Sentinel autosampler, and an LC-4C electrochemical detector. Samples (12 ul) will be run through a reverse phase micropore column at a flow rate of 0.6 ml/min and electrodetection at +0.6 V. Separation for dopamine and dopamine metabolites will be accomplished by a mobile phase consisting of 90-mM sodium acetate, 35-mM citric acid, 0.34-mM ethylenediamine tetraacetic acid, 1.2-mM sodium octyl sulfate, and 15% methanol v/v at a pH of 4.2 (Mayorga et al., 2001; Vucetic et al., 2010).

Statistical Analysis. Gene expression analysis was performed using Student T-test comparing aged matched controls to HF and HF + recovery groups. The alpha level was adjusted for the multiple brain regions surveyed using a Bonferronni correction (e.g. significance of a gene used in one brain region was p=.05; for two regions, p=0.025, for 3 brain regions p=.016). Sucrose preference, HPLC measurements, food intake, body weights, and fat pad weights were analyzed using one-way ANOVA to compare control, HF, and HF + recovery groups. Post-hoc Bonferonni Multiple Comparison Tests were used to compare

pair-wise differences between groups. Significance for these tests was set at an alpha level of p=.05.

Results

Nomenclature.

Animals that started the high fat diet at weaning are labeled “Pre-Adolescent HF”, while animals that started the high fat diet at 6 weeks of age are labeled “Adult HF.”

Body and Fat pad weights.

Body weights were taken weekly and fat pad weights were taken at the sacrifice at end of study. Within 6 weeks on HF diet, animals reached significantly higher body weight than control and remained significantly heavier than controls after a 4-week chow recovery period in both Pre-Adolescent (Figure 1A, $F(18,100)= 110.4$, $p<0.0001$) and Adult HF males ($F(20,108)=97.60$, $p<0.0001$). Pre-Adolescent HF females (Figure 1B, $F(17,95)=56.57$, $p<0.0001$) took less time to reach significantly higher body weight than Adult HF females ($F(20,115)=34.89$, $p<0.0001$). A 4-week standard chow recovery was not sufficient to normalize body weights in females. Figure 1C shows male body weights heavier in HF and HF+ recovery at experimental time points compared to their own aged matched controls in Pre-adolescent ($F(3,30)=39.59$, $p=0.0051$) and Adult HF ($F(3,28)=26.67$, $p<0.0001$). The same weight pattern is seen in females. HF and HF+ recovery Pre-adolescent (Figure 1E, $F(3,40)=40.89$, $p<0.0001$) and Adult ($F(3,54)=24.86$, $p<0.0001$) females are heavier than their aged matched control at experimental time points. Abdominal fat pad weights were taken and shown as a

percentage of body weight and compared to own aged matched controls. Pre-Adolescent males have increased fat pad mass as a percentage of body weight after high fat diet and after a 4 week standard chow replacement (Figure 1D, $F(3,34)=103.9$, $p<0.0001$). Planned comparisons shown significant increase in fat pad mass in Pre-Adolescent HF males and in Pre-Adolescent HF+recovery compared to their own aged matched controls (Figure 1D, $p<0.0001$, $p<0.05$). Adult males show a similar increase in fat pad mass after high fat diet and after a standard chow recovery (Figure 1D, $F(3,29)=79.57$, $p<0.0001$). Planned comparisons reveal Adult HF males to have significantly heavier fat pad mass versus their aged match controls (Figure 1D, $p<0.0001$) and Adult HF + recovery males were similarly increased versus their own aged matched controls (Figure 1D, $p<0.0001$). In females we see a similar increase in fat pad mass after high fat diet intake and failure of a 4 week standard chow replacement to normalize body fat pad percentage.

Pre-Adolescent females have increased fat pad mass as a percentage of body weight after high fat diet and after a 4 week standard chow replacement (Figure 1F, $F(3,30)=59.86$, $p<0.0001$). Planned comparisons shown significant increase in fat pad mass in Pre-Adolescent HF females and in Pre-Adolescent HF+ recovery compared to their own aged matched controls (Figure 1D, $p<0.0001$, $p<0.05$). Adult HF females show a similar increase in fat pad mass after high fat diet and after a standard chow recovery (Figure 1F, $F(3,32)=43.79$, $p<0.0001$). Planned comparisons reveal Adult HF females to have significantly heavier fat pad mass versus their aged match controls (Figure 1F, $p<0.0001$) and Adult HF + recovery females to have increased fat pad mass increased versus their own aged matched controls (Figure 1F, $p<0.01$).

Sucrose Preference

Sucrose preference test was administered to assess the animals' response to a natural and nutritive rewarding stimulus. All animals preferred the 4% sucrose solution over water (preference > 50%). In males, sucrose preference was significantly reduced after 12 weeks HF diet in the Pre-Adolescent animals (Figure 2A, $F(2,17)=17.58$, $p<0.0001$), yet unchanged in adult HF animals. Sucrose preference was decreased in both female Pre-Adolescent HF (Figure 2C, $F(2,16)=12.07$, $p<0.0006$) and Adult HF mice ($F(2,15)=7.497$, $p<0.0055$) after 12 wks high fat diet. Preference in all groups where sucrose preference was decreased returned to control levels after standard chow recovery period.

One-Hour Palatable Food Intake

Animals were given one-hour access to high fat/high sugar food to assess their response to intermittent palatable food. In Pre-Adolescent males, chronic HF reduced the amount of palatable food intake (Figure 3A, $F(7,39)=13.58$, $p<0.0001$). 1 hr access to control food was not changed. Post-hoc analysis shows that standard chow replacement during the experimental period or for 4 weeks prior increased palatable food intake compared to pre-adolescent animals on chronic HF (Figure 3A, $p<.001$, $p<.0001$). Female Pre-Adolescent HF animals did not significantly reduce their 1hr palatable food but they increased their intake after the 4wk standard chow replacement (Figure 3B, $F(7,41)=9.281$, $p<0.0001$). Post hoc analysis revealed an increase in 1hr palatable food intake after 4wk standard chow replacement to be statistically greater than controls (Figure 3C, $p<.01$). Both a 1wk and 4wk standard chow recovery groups were statistically higher than Pre-Adolescent HF female group (Figure 3B, $p<.001$, $p<0.0001$).

Similarly, adult HF males had significantly reduced 1hr palatable food intake compared to controls. (Figure A $F(7,38)=11.89$, $p<0.0001$). A standard chow replacement normalized 1hr intake levels after 1wk recovery and after 4wk recovery. Post hoc analysis in males revealed Adult 1wk recovery group to be statistically higher than Adult HF (Figure 3A, $p<0.0001$). Adult HF females also reduced their 1hr intake while on HF diet and normalized intake after both a 1wk standard chow recovery and a 4wk standard chow recovery (Figure 3B, $F(7,39)=16.47$, $p<0.0001$). Post hoc analysis revealed both HF+ 1wk recovery and HF+ 4wk recovery to be significantly higher than Adult HF intake in females (Figure 3B, $p<0.01$, $p<0.0001$).

Percent of daily calories eaten during the 1 hr food access was also calculated in Pre-Adolescent males (Figure 3C, $F(7,37)=7.698$, $p<0.0001$). There was a decrease in percent of daily calorie intake in Pre-Adolescent HF males that normalized after standard chow removal. Post hoc analysis revealed a statistical increase after 4 wk standard chow replacement compared to animals on a HF diet (Figure 3C, $p<0.0001$). Pre-Adolescent HF females did not significantly decrease their 1 hr palatable food's percent of daily calories, however it was significantly increased after 1wk and 4wk standard chow replacement (Figure 3D, $F(7,41)=9.907$, $p<0.0001$). Post hoc analysis revealed standard chow replacement for 1wk or 4wks was significantly higher than controls (Figure 3D, $p<0.0001$,$p<0.05$). 1wk recovery and 4wk recovery females also significantly increased their daily calorie percentage compared to Pre-Adolescent females on HF diet (Figure 3G, $p<0.0001$, $p<0.001$). Pre-adolescent HF females reached binge level intake of palatable food after 1wk and 4 wk recovery (<25% daily intake). Adult HF males had decreased percent daily intake of palatable food compared to controls and this was normalized after 1wk or 4wk standard chow recovery (Figure 3C $F(7,37)=11.62$, $p<0.0001$). Post hoc analysis revealed that a 4wk standard chow recovery leads to a

significant increase in daily percentage compared to Adult males on HF (Figure 3C, $p<0.0001$). Similarly, adult HF females had a decreased percentage of daily calories from 1hr intake compared to controls and this was normalized by 1wk or 4wk standard chow recovery (Figure 3D, $F(7,39)=16.25$, $p<0.0001$). Post hoc analysis revealed that 1wk recovery or 4wk recovery lead to significantly higher percentage of daily intake than Adult females on HF (Figure 3D, $p<0.001$, $p<0.0001$).

Gene Expression

Dopamine-related gene expression was examined within the reward circuitry [Table 1 summarizes the statistical analysis and gene expression patterns in the VTA, NAc, and PFC regions in both male diet groups compared to their aged matched controls]. All gene expression data is presented in the table as fold change values from separate aged-matched controls for HF and HF+ recovery cohorts. Only one control group for HF group is shown on graph and set to 1 for clarity. Key findings will be highlighted here. Significance was determined at a corrected alpha level of p=(0.05/# brain regions assayed). Dopamine related genes in the VTA of Pre-Adolescent males were down regulated after high fat intake and changes were more persistent than Adult HF males. Student's t-test (n=5/group) revealed in the VTA of Pre-Adolescent HF males DAT and TH mRNA were significantly decreased after HF exposure (Figure 4A, $p<0.001$, $p< 0.001$). COMT followed a different pattern; mRNA levels were elevated after 12wks HFD (Figure 4A, $p<0.01$). After a 4 week standard chow replacement, DAT and TH mRNA levels remained significantly decreased (Figure 4A; $p<0.0001$, $p<0.0001$) and COMT remained significantly elevated (Figure 4A, $p<0.01$). In the VTA of Adult HF males, DAT and TH were similarly decreased after 12 week HF exposure (Figure 4B; $p<0.05$,

p<0.01), however in these animals DAT and TH mRNA levels in Adult HF males to control levels normalized after the 4wk standard chow replacement.

In the NAc and PFC, genes important for DA signaling and DA turnover were examined (n=5/group). Pre-Adolescent HF males had a different pattern of gene expression than Adult HF males in the NAc. Figure 4C shows Pre-Adolescent HF males had increased mRNA levels of COMT and DARPP-32 after 12 wks HFD. DR1 and DR2 showed a different pattern of expression and were decreased in the NAc of Pre-Adolescent HF males (Figure 4C, p<.01, p<.01). A 4 week standard chow replacement was able to normalize COMT and DARPP-32 back to control levels in Pre-Adolescent HF male NAc. Pre-Adolescent HF male DR1 and DR2 mRNA levels remained significantly decreased after a 4 week recovery period (Figure 4C, p<.025, p<.0001). In Adult HF males a different pattern emerged. In the NAc of Adult HF, exposed mice had decreased mRNA levels of COMT and DARPP-32 (Figure 4D, p<.01, p<.01) and decreased DR1 and DR2 mRNA (Figure 4D, p<.01, p<.025) after 12wk HFD exposure. All genes examined in Adult HF males normalized to control levels after a 4 week standard chow replacement.

Gene expression of DA related genes decreased in the PFC of both Pre-Adolescent HF and Adult HF males and both were able to normalize to control levels after a 4wk recovery in most genes analyzed. Figure 4E shows decreased expression of COMT, DR1 and DR2 mRNA in Pre-adolescent HF (Figure 4E, p<.001, p<.001, p<.025) after 12wks HFD. After a 4-week standard chow replacement, mRNA levels of COMT and DARPP-32 normalized in the PFC of Pre-Adolescent HF males (Figure 4E). The Adult HF male PFC had decreased COMT, DARPP-32 and DR1 mRNA levels (Figure 4F, p<.01,p<.01, p<.0001) after 12wk HFD. COMT remained significantly decreased in

Adult HF+ recovery males (Figure 4F; p<0001); however, DR1 levels returned to control levels normalized after the standard chow recovery period. DR2 mRNA levels in the PFC remained unaffected.

The same regions and genes were examined in female mice (n=5/group). [Table 2 summarizes the statistical analysis and gene expression patterns in the VTA, NAc, and PFC regions in both female diet groups compared to their aged matched controls]. There were significant differences observed in the gene expression pattern in response to 12wks HFD and HFD followed by recovery at different developmental periods. Most notably, we observed an important sex difference in the regulation of DAT in the female VTA. DAT mRNA levels increased after 12 week HF diet (Figure 5 p<.05). Additionally in the VTA, female Pre-Adolescent HF TH mRNA levels were decreased (Figure 5A; p<.05) and COMT remained unchanged. DAT mRNA levels remained elevated (Figure 5A, p<.05) and TH mRNA remained decreased (p<.01) in Pre-Adolescent HF female VTA after a 4 week standard chow replacement. In Adult HF female VTA, we observed a similar increase in DAT expression (Figure 5B; p=.054) that recovered. COMT and TH expression were unchanged in Adult HF female VTA.

In the NAc, both Pre-Adolescent and Adult HF females had a similar pattern of disruption of dopaminergic gene expression after HF diet and diet removal. Pre-Adolescent females had decreased DR1 and DR2 receptor mRNA in the NAc (Figure 5C, p<.01, p<.01) as well as decreased COMT mRNA (Figure 5C, p<.01). A 4-week standard chow replacement was unable to normalize the expression of COMT and DR2 and they remained significantly decreased in Pre-Adolescent HF females (Figure 5C; p<.001, p<.001). Adult HF females showed a similar decrease in DR1 and DR2 (Figure 5D, p<.01; P<.001) as well as decreased DARPP-32 mRNA (Figure5D, p<.01) in the

NAc. After a 4 week standard chow replacement, DARPP-32 and DR1 expression normalized to control levels. However, DR2 mRNA remained decreased (Figure 5D, $p<.001$) in Adult HF +recovery female NAc.

Gene expression analysis in the PFC revealed further differences between Pre-Adolescent HF and Adult HF exposed females. In Figure 5E, Pre-Adolescent females showed decreased COMT, DARPP-32, DR1 and DR2 mRNA expression after 12wks HFD ($p<.001$, $p<.01$, $p<.0001$, $p<.025$, respectively) and all but COMT recovered. A 4-week standard chow replacement failed to normalize DARPP-32 (Figure 5E, $p<.025$) and left DR1 significantly increased compared to controls ($p<.025$). 12 weeks HF diet in Adult females did not alter dopaminergic genes in the PFC (Figure 5F). However, the standard chow replacement did lead to significant increases in DARPP-32 and DR1 mRNA (Figure 5F; $p<.025$, $p<.0001$) in Adult female PFC.

Dopamine and DOPAC Levels

Given the changes in gene expression for dopamine (DA) regulating genes in the VTA, DA and DA metabolites were quantified in regions that receive projections from the VTA: NAc and PFC (n=7-10/group). Figure 6 shows DA and the DA metabolite, DOPAC, measured in the NAc and PFC of males and females exposed to the HF diet at different developmental periods. In general, the NAc had higher levels of DA and DOPAC than the PFC. High fat diet altered DA levels only in Pre-Adolescent males while Adult HF males were unaffected. Dopamine in the NAc was increased after chronic HFD in Pre-Adolescent males and remained significantly increased after standard chow replacement (Figure 6A, $F(2,26)=8.267$, $p< .0.01$). DOPAC in the NAc was decreased by chronic HF in these animals and remained decreased after standard chow replacement (Figure 6A,

F(2,26)=6.127, p< 0.01). On the other hand, DA and DOPAC levels in Adult HF males NAc were unaffected by HF and HF+recovery (Figure 6A, F(2,22)=1.832, p=0.1838; F(2,22)=1.503, P=0.2444, respectively). A measure of dopamine turnover was calculated as the ratio of DOPAC:DA for all groups and brain regions. Dopamine turnover was decreased in Pre-Adolescent HF and remained decreased after recovery period (Figure 6B, F(2,26)=10.25, p< 0.001). Dopamine turnover was unaltered in Adult HF animals. In the PFC, only Pre-Adolescent male DA levels were affected by chronic HFD. Figure 6E shows the DA and DOPAC levels in the PFC of Pre-Adolescent males. One-way ANOVA revealed DA was increased compared to controls (Figure 6E, F(2,25)=5.321, p< 0.05) after 12wk HFD. A 4wk standard chow recovery period normalized DA back to control levels in this region. Pre-Adolescent HF DOPAC levels in the PFC was unaltered (Figure 6E F(2,17)=1.135, P= 0.3447). DA and DOPAC levels were not significantly different compared to controls in Adult HF.

DA, DOPAC and DA turnover were also measured in the NAc and PFC of females. In general, females DA levels seemed less affected by HFD and age of exposure in these regions. In contrast to males, female Pre-Adolescent group was unaltered by HF and HF+recovery in the NAc. DA (Figure 6C, F(2,27)=0.1211, P=0.8864) and DOPAC (Figure 6C, F(2,26)=2.840, P=0.0767) were not changed compared to controls. DA turn over was decreased in Pre-Adolescent HF+ recovery group (Figure 6D, F(2,27)=5.543, P < 0.01). Adult HF females had higher levels of DA in the NAc, but neither dopamine or DOPAC were significantly different than controls after HF or HF+recovery (Figure 6C, F(2,20)=1.132, P=0.342; F(2,20)=2.521, P= 0.1056). Dopamine turnover in the female Adult HF NAc was also unaltered compared to controls (Figure 6D, F(2,20)=0.9218, P=0.4141).

Similar to males, female Pre-Adolescent HF PFC had increased DA after 12 wks HFD that normalized after 4wk recovery (Figure 6G). One-way ANOVA revealed dopamine was significantly different than control levels and DOPAC levels were unaffected (Figure 6G, $F(2,26)=3.845$, $p < 0.05$; $F(2,21)=0.1879$, $p=0.8301$). In contrast to the NAc, PFC dopamine turnover was unaffected by 12wk HF. However it was increased in Pre-Adolescent HF+ recovery (Figure 6H, $F(2,17)=16.03$, $P< 0.0001$). Similar to Pre-Adolescent HF females, dopamine in the Adult HF PFC was significantly increased compared to controls (Figure 6G, $F(2,23)=6.839$, $P < 0.01$) and normalized after 4wk recovery period. In Figure 6H, analysis revealed DOPAC to be unaltered in Adult HF (Figure 6G, $F(2,15)=1.630$, $P=0.2288$) and dopamine turnover to be increased in Adult HF+ recovery female PFC (Figure 6H, $F(2,13)=16.67$, $P < 0.001$).

Discussion

We have investigated the age of onset and sex as critical variables in the neuro-adaptations seen after chronic high-fat diet intake and standard chow replacement. Chronic high-fat diet caused changes in sucrose preference, intake of palatable food, dopaminergic gene expression, and dopamine neurotransmitter levels. We demonstrated that it is possible to reverse the neuroadaptations and behavioral changes seen after high fat diet by a 4-week standard chow replacement. However, in some cases, we observed a pattern of persistence that depended on both age of diet onset and sex. The rodent brain continues to development after parturition and this postnatal development is highly influenced by the environment and nutritional state. We hypothesized that an earlier exposure to high fat diet would be more damaging to the dopamine reward system than if the diet was started later in life. We demonstrated an

earlier onset of high fat diet intake decreased sucrose preference and increased dopamine neurotransmitter levels while adult onset remained unaffected in our male animals. We demonstrated that high fat diet had a similar effect in females starting high fat intake early or later in life. However, there were important sex differences observed in response to diet onset. We also hypothesized that an earlier exposure to high fat diet would lead to more a more persistent phenotype than if diet was started later in life. After a 4-week standard chow replacement, behavioral changes returned to control levels, however gene expression changes and neurotransmitter level changes persisted in males beginning the diet early in life. Gene expression changes also failed to normalize in females who began the high fat diet earlier in life. Failure to normalize gene expression changes after standard chow replacement may be due to disruption during a sensitive time window from weaning to 6 weeks of age.

The two critical factors we looked to examined in response to high fat diet and high fat withdrawal were sex and age of onset. Some important sex differences were observed in body weight gain, one-hour palatable food intake, and dopaminergic gene expression. Many of the sex differences were seen in response to the high fat diet and not in the response to high fat removal. In both males and females age of diet initiation had little impact on final body weight. However, Adult HF males gained weight more rapidly than Pre-Adolescent males and obtained a significantly different body weight after only 3 weeks on HF diet. The opposite was seen in females; it took longer for Adult HF females to become significantly heavier than controls. This could be due to the protective effects higher level of estrogen in the post-pubertal period that can increase metabolism. Both males and females failed to normalize their body weight after a 4-week standard chow replacement. This fact was important to analyzing the effects of removal of the high fat diet and the response to significant weight loss. An important sex

difference occurred in the sucrose preference test where adult HF exposed males remained unaffected and females had decreased preference for sucrose. Sex has previously been shown to be important in self administration of other rewarding substances such as saccharin, cocaine and morphine (Dess et al., 1998; Dess, 2000; Carroll et al., 2002) with females having a more rapid rate of acquisition and an increase in administration. The decrease in sucrose preference is consistent with the reward deficiency model of obesity. The decrease in value natural rewards could lead to overconsumption of rewarding food in order to increase total reward levels.

To determine the risk of overconsumption in animals given chronic HF diet, we gave mice daily 1-hour access to high fat/high sugar food and measured their caloric intake. Intermittent access has been previously used to elicit binge eating in lean rodents (Corwin et al., 2004). “Bingeing” is typically defined as a bout of food intake greater than what normally would be consumed in the same period (>25% daily calories in one hour). One important sex difference we see that females given high fat diet earlier in life do not suppress their palatable food intake while on high fat diet as seen in males. This could mean that pre-adolescent exposed females lose the ability to regulate their intake when there is already calorically dense food available. Another important observed sex difference was seen in response to high fat diet removal on one-hour palatable food intake. Both males and females on the HF diet tend to reduce thier intake during the 1hr palatable food access than controls. However, after acute and long term removal of high fat diet, males normalized palatable food intake while pre-adolescent exposed females greatly increased intake and reached binge levels of eating. Indeed, binge eating is more prevalent in females than in males (Kessler et al., 2013) but this seems to be an interaction with sex and removal of HF diet. Decreased inhibitory control could be the underlying factor behind the difficulty with dieting and weight loss in women (Woods et

al., 2003). Our study has shown that chronic HF intake does not cause overconsumption when the HF is present. It is the acute removal of HF that causes an increase in palatable food consumption and females may be prone to bingeing in this state.

Dopamine is an important neurotransmitter that regulates the intake of rewarding substances. Our lab has previously published decreases in DA and opioid related genes in reward related regions after chronic HF for varying lengths of time (Vucetic et al., 2012; Carlin et al., 2013). Changes in dopaminergic genes have been seen in many obese rodent models, but few studies have looked at this system after a standard chow replacement period. Interesting age and sex differences were revealed both in the response to the HF diet and after a 4 week recovery period. In females, we observed an interesting sex difference in the VTA. Females given chronic high fat diet had increased DAT expression while males had decreased DAT levels. The difference in DAT expression would oppositely affect levels of dopamine in the synapse and may be important for some of the behavioral differences observed. Sex differences in basal DAT expression have previously been observed in both humans (Lavalaye et al., 2000) and animals (Rivest et al., 2005) and revealed DAT levels to be overall higher in females. Our lab has previously published that high fat diet beginning at birth regulated DAT levels through DNA methylation at the promoter region (Carlin et al., 2013). Further studies are needed to understand how sex hormones and epigenetic machinery interact to regulate gene expression in response to high fat diet and high fat replacement.

The second factor we found to be important in regulating the reward system's response to high fat diet and HF replacement was age of onset. We predicted that an earlier age of onset for high fat diet would be more damaging to the dopamine reward system than adult onset. Sucrose preference test in our study was one example where

age of HF diet onset was important. The pre-adolescent time period is an important window for programming the taste for palatable foods (Teegarden et al., 2009). We found a decrease in sucrose preference for pre-adolescent HF males while adult HF males remained unaffected. Sucrose preference is a broad measure of natural reward intake. Decrease in sucrose consumption is considered an animal model of anhedonia. The decrease in sucrose preference is consistent with the hypo reward hypothesis of obesity. Obese patients show decreased activity in reward related regions when ingesting a palatable solution (Stice et al., 2010). We also observed normalization of sucrose preference after a 4 week standard chow recovery period. Normalization is also observed in gastric bypass patients who show increases in sucrose preference after surgery (Bueter et al., 2011). The decrease in sucrose preference seems to be caused by the presence of the HF diet and not the factor of obesity itself. This factor emerges in non-diet induced obese rodent models such as the OLETF CCK-1 knockout rats that become obese on control chow. OLETF rats show increased preference for sucrose preference compared to controls (Marco et al., 2012). This could be due to high fat diet neuroadaptations causing decreases in reward value or increases in satiety levels.

We predicted that an earlier high fat diet would cause changes in the dopamine reward system that would persist throughout a standard chow recovery period because of possible programming effects that can occur during a vulnerable developmental window. Pre-Adolescent and Adult HF animals had a similar gene expression response to the high fat diet. It was in the standard chow replacement period where we observed differences in the ability to normalize between age groups. For example, a 4 week standard chow recovery period was unable to normalize the gene expression levels COMT, DAT, and TH in the VTA in animals who began HF diet earlier in life. In the post synaptic region of the NAc, animals that began high fat diet earlier in life had decrease

expression of DR1 and DR2. This is consistent with the hypodopaminergic hypothesis of obesity. Further, a 4 week standard chow recovery was unable to normalize DR1 and DR2 in the NAc of these animals. Adult HF exposure had similar decreases in dopamine receptors but normalized expression after the recovery period. Further, pres-adolescent exposure to high fat caused increases in DA that persisted in the NAc after 4 week standard chow replacement while adult exposure DA levels remained unchanged. Early life exposure to high fat also seemed to be more detrimental in females. Early life exposure to high fat decreased expression levels of COMT, DARPP-32, DR1, and DR2 in both the NAc and PFC. A 4 week standard chow replacement was unable to normalize genes in the NAc, however DR1 and DR2 both normalized in the PFC. Interestingly, the PFC of the Adult HF females exposure group was unaffected by high fat diet. Decreases in DR2 were seen in response to high fat diet and remained decreased after a recovery period in animals exposed to diet earlier in life. Low dopamine receptor D2 levels are implicated in obesity, drug addiction and impulsive behaviors (Wang et al., 2001). Decreased DRD2 in reward regions predicts reward dysfunction and binge-like behavior (Johnson & Kenny., 2010) and decreases in gene expression in diet induced obesity could leave animals more prone to overconsumption. We see from these results that age of onset is an important factor when examining gene expression changes and persistence after diet manipulation. It suggests the existence of a vulnerable period that when manipulated, can alter the response to high fat diet in adulthood.

In summary, we have shown that chronic HF diet leads to changes in dopamine circuitry and reward consumption and that it is possible to reverse these changes before body weight normalization with a 4 week standard chow replacement. We revealed chronic HF diet leads to altered dopaminergic gene expression, neurotransmitter levels,

and dopamine turnover in a sex and age dependent manner. Moreover, the reversal of these changes is more difficult in animals who received high fat diet earlier in life. Women and adults who were obese as children find it more difficult to lose weight then the rest of the population. Altered dopamine circuitry that persists during “dieting” could be behind high failure rates seen in these populations. Further studies are needed to examine how these populations are more vulnerable to high fat diet circuitry changes and which specific changes are behind the overconsumption of palatable food in obesity. Other neurotransmitters, such as the endogenous opioid system, also regulate intake and the “liking” of natural rewards. It has been shown that mu opioid receptors (MOR) levels are decreased on high fat diet and this is associated with increase in epigenetic marker levels, such as DNA methylation at promoter region (Vucetic et al., 2011). It is possible that epigenetic machinery may be altered by high fat diet during the vulnerable window from weaning to 6 weeks of age, therefore programming the obesity phenotype into adulthood. Differences in the ability of these changes to normalize may be behind the difficulty in weight loss and the relapse to over consumption that occurs in many obese individuals. Understanding how age of onset and sex interact with gene expression changes after removal of HF diet will help us predict who is most vulnerable to overconsumption and point us in the right direction for better obesity interventions.

Figure and Table Legends

Table 1. Gene Expression Summary and Statistics in Males. Gene expression results for male ventral tegmental area (VTA), prefrontal cortex (PFC), and nucleus accumbens in both Pre-Adolescent and Adult HF males. Summary of fold change, significance and p values are presented for HF and HF +recovery groups as compared to their age matched control.

Table 2. Gene Expression Summary and Statistics in Females. Gene expression results for male ventral tegmental area (VTA), prefrontal cortex (PFC), and nucleus accumbens in both Pre-Adolescent and Adult HF females. Summary of fold change, significance and p values are presented for HF and HF +recovery groups as compared to their age matched control.

Figure 1. 12 week exposure to high fat diet causes increase in body and fat pad weights and these increases are not normalized by a 4 week standard chow replacement. Body weights (n=10/group) were measured weekly in Pre-adolescent (blue triangles) and Adult HF animals (green squares) males (A) and females (B) and shown in grams. Red dashed line depicts time on HF diet, blue dashed line depicts time of standard chow recovery. Control animals (black triangles) remained on standard chow throughout the experiment. C) Bar graph of end point body weights of control (white bar), HF (black bar), and HF+ recovery (horizontal striped bar) males in grams. D) Normalized Abdominal Fat Pad weights in males shown as percentage of end point body weight E) Bar graph of end point body weights of control (white bar), HF (grey bar), and HF+

recovery (horizontal striped bar) females in grams. F) Normalized abdominal fat pad weights in females shown as percentage of end point body weight.

Post hoc comparisons shown at alpha levels *p<.05, **p<.01, ***p<.001, ****p<.0001 significantly different from aged matched control group.

Figure 2. Sucrose preference is decreased after high-fat diet (HFD) exposure in Pre-Adolescent HF males and females and recovers after standard chow replacement. Percent preference for 4% sucrose was evaluated in control (white bars) HF (solid bars) and HFD + recovery (striped bars) Pre-Adolescent and Adult HF age groups (n=8/group). Males are shown in black (A), females are shown in grey(B). Post hoc comparisons are shown at alpha levels *p<.05, **p<.01, ***p<.001 significantly different from own control group.

Figure 3. Chronic HFD causes decrease intake of palatable food during one-hour challenge. Standard chow replacement during experiment or 4 weeks caused an increase in palatable food intake. Intake during one-hour palatable food access was measured in control (white bars) HF (solid bars) and HF + 1wk recovery (dotted bars) and HF + recovery (horizontal striped bars) for Pre-Adolescent and Adult HF males (A) and females (B). Percent of daily calories eaten during the 1 hr palatable food access was calculated for both male (C) and female (D) age groups (n=3/group for 1hr control chow access and n=9/group for 1hr palatable food access. Post hoc planned comparisons shown at alpha levels *p<.05, **p<.01, ***p<.001 significantly different from own control group or ##p<.01, ###p<.001, ####p<.0001 significantly different from HF group.

Figure 4. Chronic high-fat diet (HFD) alters dopamine related gene expression in males. Pre-Adolescent HF males were less able to normalize gene expression in

examined reward regions of the brain. Gene expression was measured in the ventral tegmental area (VTA;A,D), nucleus accumbens (NAc; B,E), and prefrontal cortex (PFC; C,F) of control (white bars), HFD (solid bars) and HFD + recovery (striped bars) Pre-Adolescent and Adult HF male age groups (n=5/group). HF and HF + recovery groups are shown as fold change from their own aged matched controls. Only one control group (15wk) is shown and set to 1 for clarity. Post hoc planned comparisons shown at alpha levels *p<.05, **p<.01, ***p<.001, ****p<.0001 significantly different from own control group.

Figure 5. Chronic high-fat diet (HFD) and HF+ recovery alters dopamine related gene expression differently in Pre-Adolescent and Adult HF females. Gene expression was measured in the ventral tegmental area (VTA; A,D), nucleus accumbens (NAc; B,E), and prefrontal cortex (PFC; C,F) of control (white bars), HFD (solid bars) and HFD + recovery (striped bars) Pre-Adolescent and Adult HF female age groups (n=5/group). HFD and HF + recovery groups are shown as fold change from their own aged matched controls. Only one control group (15wk) is shown and set to 1 for clarity. Post hoc planned comparisons shown at alpha levels *p<.05, **p<.01, ***p<.001, ****p<.0001 significantly different from own control group.

Figure 6. Dopamine levels are increased after chronic HFD in Pre-Adolescent HF males. Dopamine levels in the NAc do not normalize after 4wk recovery. Dopamine (DA) and dopamine metabolite 3,4-Dihydroxyphenylacetic acid (DOPAC) and dopamine turnover were measured in the NAc (A-D) and PFC (E-H) of control (white bars), HF (solid bars) and HF + recovery (striped bars) Pre-Adolescent and Adult HF age groups (n=8-10/group). DOPAC:DA ratio is a measure of dopamine turnover. Males are shown in black and females in grey. *p<.05, **p<.01 significantly different from control group.

Tables and Figures

Table 1. Gene Expression Summary and Statistics in Males

GENE	Pre-Ad HF Males fold change	P VALUE	Pre-Ad+recov Males fold change	P VALUE
VTA				
COMT	1.48	.0010	2.013	.0077
DAT	0.48	.0004	0.342	<.0001
TH	0.46	.0001	0.471	<.0001
NAC				
COMT	1.987	.0002	1.46	.6527
DARPP32	1.87	.0007	1.25	.0836
DRD1	0.580	.0021	0.719	.0085
DRD2	0.672	.0078	0.405	<.0001
PFC				
COMT	0.485	.0010	1.267	.0167
DARPP32	0.605	.0491	1.661	.0406
DRD1	0.485	.0010	1.139	.3756
DRD2	0.313	.0069	1.149	.7138
GENE	**Adult HF Males fold change**	**P VALUE**	**Adult HF+ recov Males fold change**	**P VALUE**
VTA				
COMT	0.94	.5670	0.84	.0128

DAT	0.674	.049	0.98	.7538
TH	0.657	.0083	1.06	.614
NAC				
COMT	0.736	.0013	0.846	.164
DARPP32	0.575	.0011	1.262	.1945
DRD1	0.638	.0025	1.284	.0474
DRD2	0.644	.0078	1.029	.8949
PFC				
COMT	0.662	.0076	0.520	<.0001
DARPP32	0.543	.0047	1.145	.4038
DRD1	0.136	<.0001	0.838	.0879
DRD2	1.538	.1629	1.696	.2261

Table 2. Gene Expression Summary and Statistics in Females

GENE	Pre-Ad HF Females fold change	P VALUE	Pre-Ad HF+ recovery Females fold change	P VALUE
VTA				
COMT	1.195	.0202	2.633	.0352
DAT	1.76	.0304	1.46	.0104
TH	0.558	.0157	0.446	.0004
NAC				
COMT	0.5708	.0011	0.682	.0007
DARPP32	0.799	.2061	0.343	<.0001
DRD1	0.256	.0030	2.95	<.0001
DRD2	0.483	.0042	0.346	.0004
PFC				
COMT	0.496	.0001	0.657	.0214
DARPP32	0.608	.0014	0.704	.016
DRD1	0.338	<.0001	2.01	.0108
DRD2	0.609	.0195	2.43	.0722
GENE	**Adult HF Females**	**P VALUE**	**Adult HF+ recov Females**	**P VALUE**
VTA				
COMT	1.06	.5672	1.384	.0498
DAT	1.28	.0546	0.692	.0219

TH	0.937	.3179	0.907	.2155
NAC				
COMT	3.97	.0172	0.288	.0003
DARPP32	0.456	.0017	0.639	.1017
DRD1	0.374	.0023	0.833	.0355
DRD2	0.178	<.0001	0.353	.0019
PFC				
COMT	0.807	.0438	0.726	.0348
DARPP32	1.057	.8899	1.468	.0186
DRD1	1.129	.5851	3.08	<.0001
DRD2	2.49	.0897	1.32	.4875

Figure 1

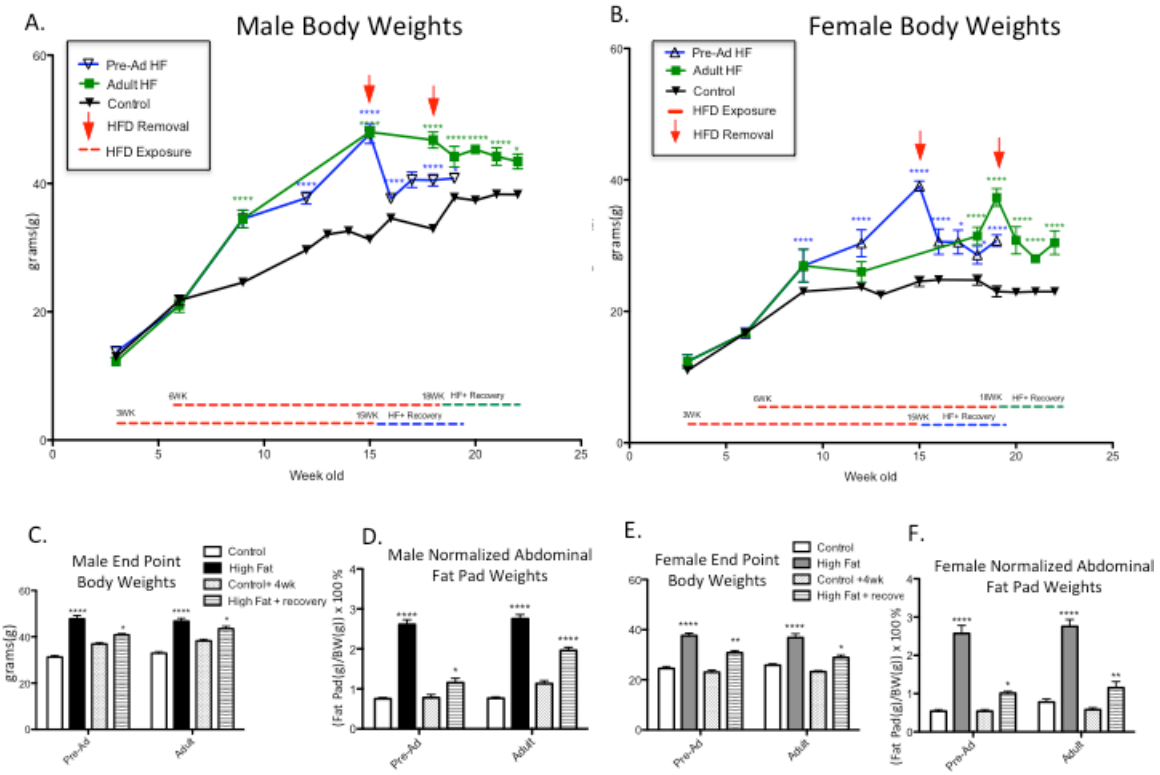

A.
Male Body Weights
Pre-Ad HF
Adult HF
Control
HFD Removal
HFD Exposure
grams(g)
Week old
B.
Female Body Weights
C.
Male End Point Body Weights
Control
High Fat
Control+ 4wk
High Fat + recovery
grams(g)
D.
Male Normalized Abdominal Fat Pad Weights
(Fat Pad(g)/BW(g)) x 100 %
E.
Female End Point Body Weights
F.
Female Normalized Abdominal Fat Pad Weights
Pre-Ad
Adult

Figure 2 Sucrose Preference

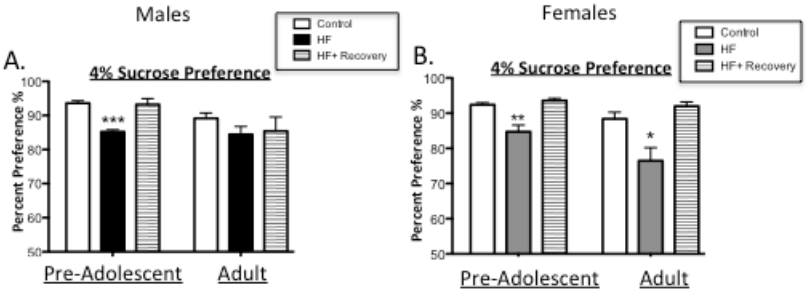


Figure 3

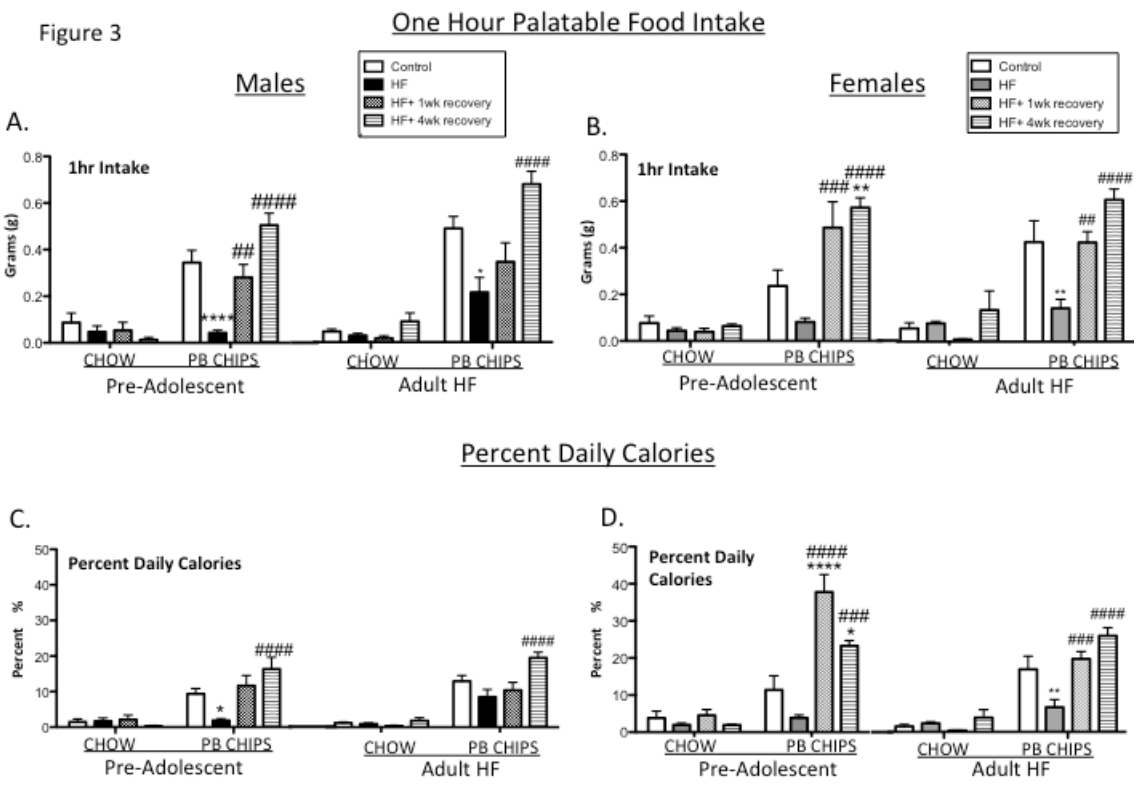

One Hour Palatable Food Intake
Males
Females
Control
HF
HF+ 1wk recovery
HF+ 4wk recovery
A.
B.
1hr Intake
Grams (g)
CHOW
PB CHIPS
Pre-Adolescent
Adult HF
Percent Daily Calories
C.
D.
Percent %

Figure 4.

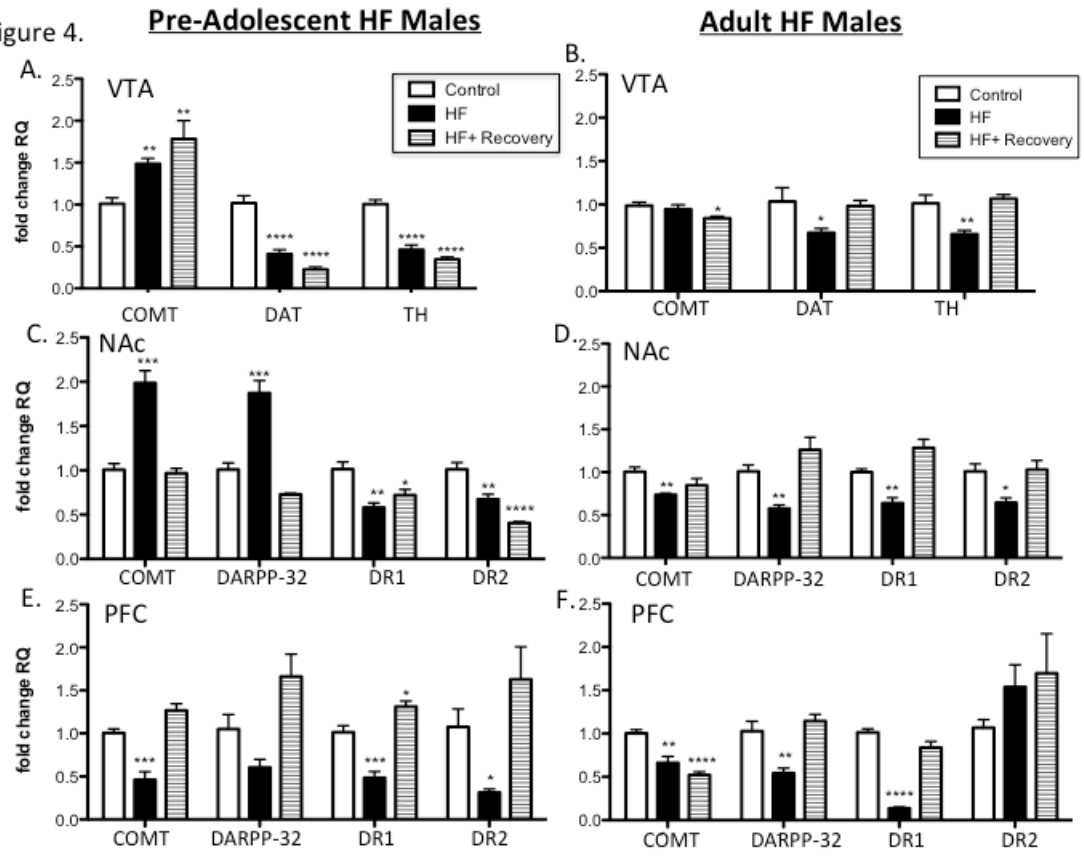

Pre-Adolescent HF Males
Adult HF Males
A.
VTA
B.
VTA
C.
NAc
D.
NAc
E.
PFC
F.
PFC
Control
HF
HF+ Recovery
fold change RQ
COMT
DAT
TH
DARPP-32
DR1
DR2

Figure 5.

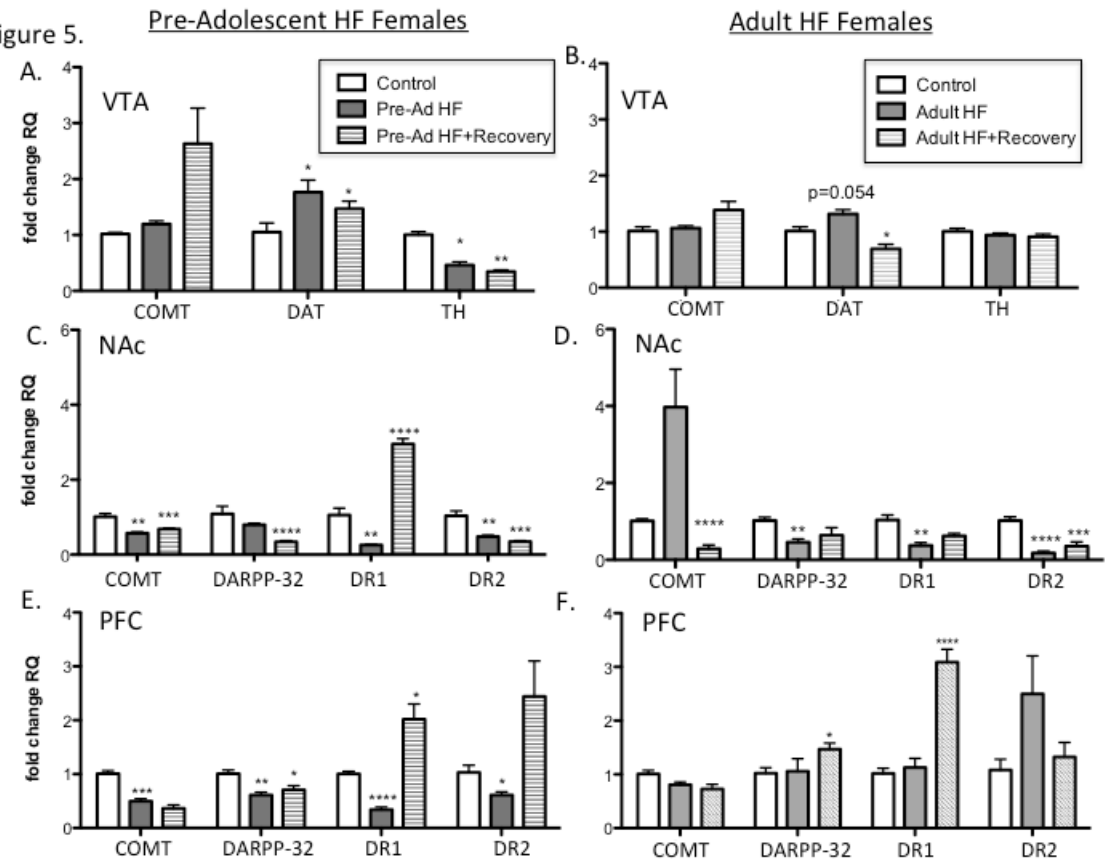
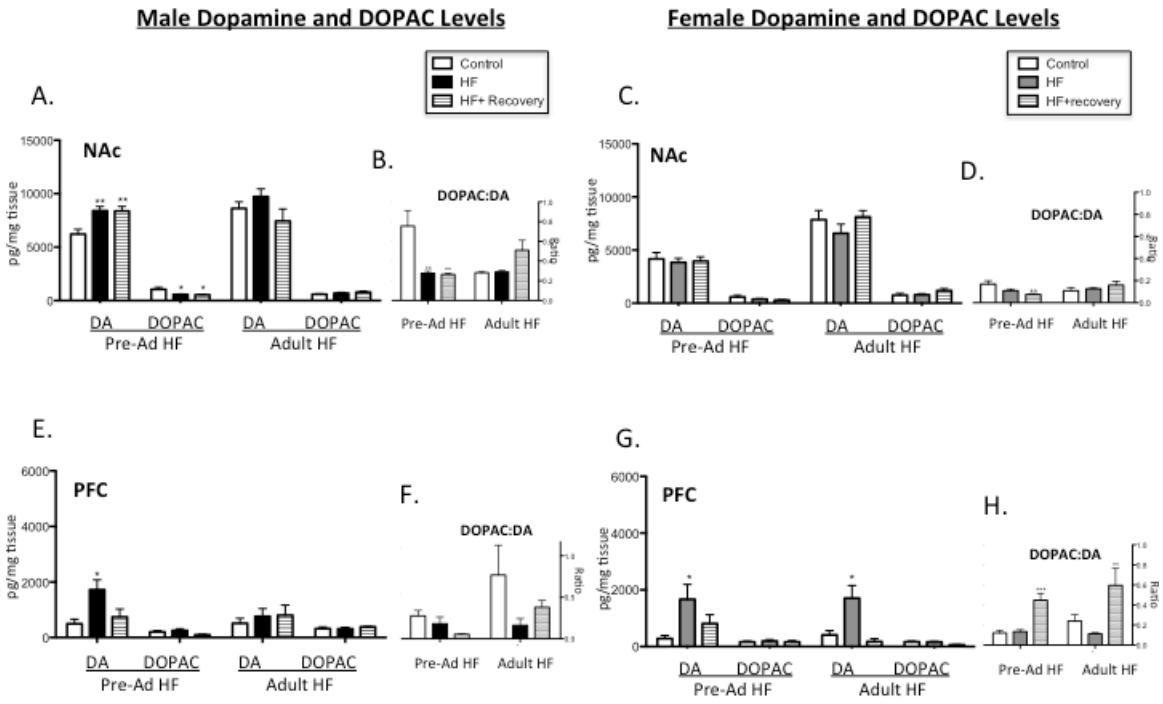

Pre-Adolescent HF Females
Adult HF Females
A.
VTA
Control
Pre-Ad HF
Pre-Ad HF+Recovery
fold change RQ
COMT
DAT
TH
B.
VTA
Control
Adult HF
Adult HF+Recovery
p=0.054
COMT
DAT
TH
C.
NAc
fold change RQ
COMT
DARPP-32
DR1
DR2
D.
NAc
COMT
DARPP-32
DR1
DR2
E.
PFC
fold change RQ
COMT
DARPP-32
DR1
DR2
F.
PFC
COMT
DARPP-32
DR1
DR2

Figure 6.


Male Dopamine and DOPAC Levels
Female Dopamine and DOPAC Levels
A.
B.
C.
D.
E.
F.
G.
H.
Control
HF
HF+ Recovery
HF+recovery
NAc
PFC
DOPAC:DA
pg/mg tissue
Ratio
DA
DOPAC
Pre-Ad HF
Adult HF

CHAPTER 4: Voluntary wheel access and highly palatable diet regulate gene expression and behavior in the central reward system

Jesse L. Carlin, Zhe Ying, Fernando Gomez-Pinilla, Teresa M. Reyes

Introduction

The rise in obesity rates in the United States has paralleled the increasing availability of energy dense foods (Flegal et al., 2012). Palatable foods are often consumed because of their hedonic properties after bodily energy requirements are already met. Palatable foods increase dopamine in the reward regions of the brain and over a long period of time, consumption cause neuroadaptations that alter reward driven behaviors. For example, consumption of high fat diet does not just drive obesity, but drives binge-like eating behavior (Corwin, et al. 1998, Lucas et al., 1989), decreases dopamine receptor levels (Johnson & Kenny, 2010), decreases neurotransmitter levels (Carlin et al., 2013; Geiger et al. 2007), and decreases natural reward intake (Carlin et al., 2013; Vucetic et al., 2011). Part of the first line of treatment for obesity is daily exercise. Exercise provides individuals with both positive metabolic effects (Rothman et al. 2012) and cognitive benefits (extensively reviewed in Chang et al., 2012). The majority of the central benefits of exercise have focused on the hippocampus; here we examine the response of reward and feeding regions of the brain to exercise. Similar to palatable food, exercise also activates reward regions of the brain and long-term wheel running has been shown to produce neuroadaptations that may overlap with those seen in obesity. Exercise is rewarding and rodents will work for access to a running wheel (Brene et al., 2007). Wheel running has also been shown to modulate reward behaviors such as decreased conditioned place preference to cocaine (Thanos et al., 2010).

Because exercise is rewarding, it is unknown whether exercise can prevent the dopamine dysfunction seen in the reward regions of the brain after chronic high fat diet.

We looked to examine first if exercise can affect the expression of reward related genes in the prefrontal cortex after short term exercise exposure. Previous studies have shown exercise can enhance cognitive function (Winter et al., 2007), and improve symptoms of depression (Lawlor & Hopker, 2001). Access to a running wheel promotes neurogenesis in the brain (van Praag et al., 1999) and increases expression of BDNF mRNA (Gomez-Pinilla et al., 2011) and epigenetic related genes, such as DNMTs and HDACs (Abel and Rissman, 2013). Research on the beneficial effects of exercise has primarily focused on the hippocampus and brain derived neurotrophic factor (BDNF) as the central mediator of brain plasticity in this region (Gomez-Pinilla et al., 2002; Hopkins et al., 2010). We look to extend these studies by examining the effect of acute exercise on reward related genes in the prefrontal cortex.

The interaction of exercise and high fat diet at the level of feeding and neural substrates of reward is currently unknown. Specifically are the generally beneficial effects of exercise on the brain able to prevent the negative effects of high fat diet, such as dysfunctional gene expression changes and neuro inflammation in the reward system and hypothalamus of mice. Exercise may be able to act as a reward replacement and reduce the risk to over consume palatable foods. Exercise also has the ability to recruit epigenetic mechanisms, such as DNA methylation, that could prevent the neuroadaptations that occur after high fat intake. Exercise has previously been shown to impart resilience to the molecular effects of stress on the brain (Haack et al., 2008; Gerecke et al., 2013); however, studies have been mixed with the behavioral outcomes of exercise. Voluntary wheel running has both anxiety producing (Burghardt *et al.* 2004;

Fuss *et al.* 2010a,b) and anti-anxiety (Dishman *et al.* 1996; Salam *et al.* 2009) behavioral effects in rodent models. We performed behavioral analysis to assess anxiety levels in our model of high fat diet and exercise. To examine if the changes that occur after long term exercise are inhibited by concurrent access to high fat, we gave male mice access to a running wheel and a high fat /high sugar diet for 6 weeks. Four experimental groups (Sedentary-Control, Sedentary-HF, Exercised-Control, and Exercised-HF) were evaluated for changes in gene expression in brain areas that regulate reward and feeding behavior. Expression of dopaminergic, epigenetic, and inflammatory related genes in the nucleus accumbens (NAc), prefrontal cortex (PFC), and hypothalamus (HYP) were examined. Behavioral assays (sucrose preference, elevated plus maze and open field) were performed to examine if either exercise or palatable diet had the ability to modulate sucrose preference or anxiety-like behavior. Understanding the mechanisms of how high fat diet and exercise elicit neuroadaptations in the reward system will be important for future weight loss studies and obesity treatments.

Materials and Methods

Animals. C57BL/6 mice approximately 9 weeks of age were singly housed in standard polyethylene cages in an environmentally controlled room (22–24°C) with a 12 h light/dark cycle. For the first experiment, mice either remained sedentary (n=6) or were given two weeks access to a voluntary running wheel in their home cages (n=12). These mice had ad lib access to standard control diet (#5755; 18.5% protein, 12% fat, 69.5% carbohydrate, Research Diets, New Brunswick, NJ). After two weeks animals were euthanized by carbon dioxide overdose, followed by cervical dislocation; a method recommended by the Panel on Euthanasia of the American Veterinary Medical

Association. For the second experiment, mice had ad lib access to either standard control diet (#5755; 18.5% protein, 12% fat, 69.5% carbohydrate) or a western-style diet (# D12079B, 17% protein, 40% fat, 43% carbohydrate, Research Diets, New Brunswick, NJ). A subset of mice from each dietary group also received access to a voluntary running wheel in their home cages, generating 4 experimental groups of sedentary + CTRL diet, sedentary + HF diet, exercise + CTRL diet, and exercised + HF diet (n=10/group). Sucrose preference was measured at baseline, 4 weeks, and 6 weeks; while EPM and OF tests were measured only at 6 weeks. At the end of 6 weeks access to exercise and/or high fat diet, animals were euthanized by carbon dioxide overdose, followed by cervical dislocation. Animal housing and behavioral experiments took place at University of California at Los Angeles. Flash frozen brain samples were shipped to University of Pennsylvania for further analysis. Experiments were performed in accordance with the United States National Institutes of Health Guide for the Care and Use of Laboratory Animals. The UCLA Chancellor's Animal Research Committee approved all procedures used in this study.

Elevated Plus Maze. The elevated plus maze (EPM) test was carried out according to a previously established protocol (Walf and Frye, 2007 and Bhatia et al., 2011). The EPM apparatus was made of laminated wood and consisted of 2 opposing open arms (10×50 cm) and 2 opposing closed arms (10×50 cm with 30 cm high walls). The maze was placed 60 cm above the floor. White curtains surrounded the maze and behavior was recorded by an overhead video camera. Each mouse (n=12) was placed in the middle of the maze facing the open arm that faced away from the experimenter. The video camera recorded the time each mouse spent in each of the arms over a period of 5 min. A closed arm entry was counted when the mouse placed all four paws in a closed arm. An open arm entry was recorded when the mouse placed all four paws in an open arm or

when the mouse's hind-limbs were placed in the central area of the maze and both fore-limbs in an open arm with its head protruding into the open arm.

Open Field Test. The open field test was performed in a 1.2 m diameter circular tank with 60 cm walls. An inner circle, 80 cm in diameter, was marked on the tank floor to serve as a central arena. Testing began when each mouse was placed in the middle of the central arena and allowed to explore the field for 10 min. Mouse behavior (n=12) was recorded by an overhead camera. Measurement included time spent and number of entries in central arena (as described in Bhatia et al., 2011)

Sucrose Preference. Mice were individually housed (n=8-10/group) in standard cages for 3 days where one bottle with 200 ml of 4% sucrose solution (w/v), another with 200 ml of tap water and house chow ad libitum were available. Sucrose (ml), water (ml), and food consumption (g), were measured and the placement of the bottles were reversed daily. Preference was calculated using the measurements from the last 2 days only as follows: preference % = [(sucrose consumption/sucrose + water consumption) × 100]. Sucrose preference test was performed at baseline, 4 week diet and/or running wheel access, and 6 week diet and/or running wheel access.

Genomic DNA and Total RNA isolation from the brain. Brains were rapidly removed after behavioral tests, flash frozen and stored at -80C. Dissections were made on ice and regions of interest were placed in RNAlater (Ambion, Austin, TX) Brain dissections to isolate the prefrontal cortex and hypothalamus were performed as previously described (Vucetic et al. 2010, Reyes et al. 2003) Genomic DNA and total RNA were isolated simultaneously using AllPrep DNA/RNA Mini Kit (Qiagen).

Gene expression analysis by quantitative Real-Time PCR. For each individual sample, 500ng of total RNA was used in reverse transcription using High Capacity

Reverse Transcription Kit (ABI, Foster City, CA). Expression of target genes was determined by quantitative RT-PCR using gene specific Taqman Probes with Taqman gene expression Master Mix (ABI) on the ABI7900HT Real-Time PCR Cycler. Gene probes are listed in supplemental material. Relative amount of each transcript was determined using delta CT values as previously described in (Pfaffl 2001). Changes in gene expression in the prefrontal cortex (n=6-8) and hypothalamus (n=2-6) were calculated against an unchanged GAPDH standard.

Methylated DNA Immunoprecipitation (MeDIP) Assay. MeDIP assay was performed using MagMeDIP kit (Diagenode, Denville, NJ). Methylated DNA was immunoprecipitated using 15ul of magnetic beads coated with anti-5methylcytidine antibody (Diagenode) or mouse pre-immune serum. Enrichment in MeDIP fraction was determined by quantitative RT-PCR using ChIP-qPCR Assay Master Mix (SuperArray) on the ABI7900HT Real-Time Cycler. For all genes examined, primers were obtained from SuperArray (ChIP-qPCR Assays (-01) kb tile, SuperArray) for the amplification of genomic regions spanning the CpG sites located approximately 300-500 bp upstream of the transcription start sites. MeDIP results were expressed as fold enrichment of immunoprecipitated DNA for each site. To calculate differential occupancy fold change (% enrichment), the MeDIP DNA fraction CT values were normalized to Input DNA fraction CT values.

Global Genomic DNA Methylation. Changes in genomic DNA methylation were analyzed using LUminometric Methylation Assay (LUMA). Methylation sensitive and insensitive restriction enzymes (HpaII and MspI, respectively) each digested 500ng DNA with EcorI as an internal control (all enzymes from New England Biolabs, Beverly, MA) in Tango Buffer (33mm Tris-acetate, pH 7.9, 10mM Mg-acetate, 66mM K-acetate, 0.1

mg/ml BSA) purchased from Fermentas (Fermendtas Scandanavia, Stolkholm) for 4 hours at 37 degrees Celcius. 20ul of each digestion was mixed with 20ul “Annealing Buffer” and transferred to Pyrosequencing plates. DNA quantification was preformed using a polymerase extension assay by the Pyrosequencing ^{tm} platform. PSQ ^{tm} 96 SNP reagents for pyrosequencing were purchased from Biotage (Biotage AB, Uppsala, Sweden) and RediPlate ^{tm} 96 PicoGreen kit from Molecular probes (Eugene, Oregon). Peak height results were obtained to calculate methylation percentage as follows: [1-((HpaII/EcoRI)/(MspI/EcoRI))] x 100.

Statistical Analysis. In experiment one, Student t tests were performed to analyze gene expression, promoter methylation, and global methylation in short term exercise exposure (2wk) compared to sedentary controls. Statistical significance was set an an alpha level of $p<0.05$. In experiment two, two-way ANOVA was used to evaluate the effects of both diet and exercise and statistically significant main effects were presented at an alpha level of $p<0.05$. Planned comparisons were performed to compare the effect of high fat diet, the effect of exercise, and whether exercise could mitigate the high fat driven response. Statistical significance was set an alpha level of $p<0.05$.

Results

Short term access to wheel was able to alter gene expression and DNA methylation in prefrontal cortex.

In the first experiment, mice had continuous access to voluntary running wheel for two weeks in their home cages and were maintained throughout on control diet. Gene expression analysis of dopamine receptor D1 (DR1), dopamine receptor D2 (DR2) and mu opioid receptor (MOR) was performed in prefrontal cortex samples. Two week access to a wheel caused decreased expression of DR1 and MOR in the cortex (Figure 1A $p<0.05$, $p<0.05$). DR2 expression was unaffected by short term exercise exposure. Quantification of DNA methylation at promoter regions of genes affected by short term exercise was performed. Fold change from input is presented and ACTIN was used as a control gene for comparison. Promoter DNA methylation was increased by exercise exposure for both DR1 and MOR genes (Figure 1B, $p<0.05$, $p<0.05$). Global methylation of genomic DNA was unaltered by a two week exercise exposure.

Access to running wheel and high fat diet for 6 weeks caused changes in body weight and reward behavior.

In the second experiment, sedentary controls, sedentary + HF, exercised, and exercised + HF animals were generated and body weights were taken at the end of the experiment. High fat diet (HF) alone caused animals to gain a significant amount of body weight compared to sedentary controls. For body weight at end of experiment there was a significant interaction between diet and exercise (Figure 2A, $F(1,36)=6.37$, $p=0.016$). There was a main effect of the high fat diet on increasing body weight (Figure 2A, $F(1,36)=47.96$, $p<0.0001$), however weight gain in the exercised group was significantly less than in the sedentary group (Figure 2A, $p<0.01$). Body weight gain was calculated from start of experiment (baseline) up until 6 weeks and an interaction between diet and

exercise was observed (Figure 2B, $F(1,36)=4.9$, $p=0.03$). Similar to final body weight, a main effect of diet was observed (Figure 2B, $F(1,36)=47.2$, $p<0.0001$), such that all high fat fed animals gained more weight, however the exercised + HF animals gained significantly less then the sedentary HF animals (Figure 2B, $p<0.01$).

Anxiety-like behaviors were measured in these animals. There was no difference in time spent in the open arm of the elevated plus maze between experimental groups (Figure 3A), nor in the time spent in the open arena during the open field test (Figure 3B). Sucrose preference was measured at baseline, 4 weeks, and 6 weeks of the experimental period. At baseline, there was no difference between experimental groups. At 4 weeks there was a significant main effect of both HF diet (Figure 3C, $F(1,36)=22.9$, $p<0.0001$) and exercise (Figure 3C $F(1,36)=7.32$, $p=.01$). At 6 weeks, sucrose preference remained diminished in sedentary + HF, exercised, and exercised + HF groups. There was an interaction between diet and exercise at this time point (Figure 3C, $F(1,36)=9.86$, $p=.003$). At 6 weeks there was a significant main effect of diet (Figure 3C, $F(1,36)=22.5$, $p<0.0001$).

Exercise was unable to reverse gene expression changes in reward related regions.

Gene expression was analyzed in both the nucleus accumbens (NAc) and prefrontal cortex (PFC). In the NAc, there was a significant main effect for diet which increased the expression of both DR1 and DR2 (Figure 4A, $F(1,16)=35.11$, $p<0.0001$, $F(1,16)=4.62$, $p=0.04$). Exercise alone did not alter expression of DR1 and DR2. Expression of DNA methyltransferase 1 (DNMT1) was also examined in the NAc and significant interaction of diet and exercise was revealed (Figure 4A, $F(1,16)=35.11$, $p<0.0001$). There was a main effect of exercise which increase DNMT1 expression

(Figure 4A, F(1,16) 13.06, p=0.002). Planned comparisons show DNMT1 to be elevated after high fat diet compared to sedentary controls (Figure 4A, p<0.05). DNMT1 levels are also significantly elevated in exercised animals compared to exercise + HF (Figure 4A, p<0.05). In the PFC, DR1 expression levels had a non significant trend in the interaction between diet and exercise (p=.1). DR2 expression had a similar non-significant trend for interaction of diet and exercise (p=0.1). However, there was a significant main effect of diet for DR2 expression in the PFC (Figure 4B, F(1,28)=4.62, p=0.04). DNMT1 expression was unaltered by diet and exercise in the PFC at this time point.

Exercise had differing effects on neuro-inflammatory markers increased by high fat diet in the PFC and hypothalamus

Expression levels of neuro-inflammatory related genes were examined in both the PFC and the hypothalamus. Table 1 provides detailed statistical analysis of genes examined in the PFC, and important results will be highlighted here. Intake of HF diet caused a significant increase in inflammatory related genes chemokine (C-C motif) ligand 2 (CCL2), C-C chemokine receptor type 2 (CCR2), C-X-C motif chemokine 10 (CXCL10) as compared to sedentary controls (Figure 5A, F(1,17)=17.7, p<0.001, F(1,16)=4.89, p<0.05, F(1,16)=4.63, p<0.05, respectively). Exercise alone had no effect and exercise in combination with high fat diet normalized these genes to controls levels. Post- hoc analysis revealed a significant difference between sedentary + HF and exercise + HF groups for CCL2, CCR2, and CXCL10 (Figure 5A, p<0.001, p=0.01, p=0.01). Intake of HF diet in sedentary animals also caused a significant increase of inflammatory related genes interleukin-6 (IL-6), and COX-2 as well as a trending main effect of diet in prostaglandin synthase E1 (PGSE-1) in the PFC (Figure 5B, F(1,13)=11.74, p<0.01, F(1,19)-4.88, p=0.04, p=0.07). There was a significant decrease

in these inflammatory markers when exercise and high fat were combined in PGSE-1 expression levels (Figure 5A, p=0.01). Gene expression examine in the hypothalamus is considered preliminary because of the small sample size in the high fat group (n=2). Table 2 lists the detailed statistical findings of genes examined in the hypothalamus. Important results will be highlighted here. In the hypothalamus, there was a significant main effect of high fat diet on neuro-inflammatory genes CCL2, suppressor of cytokine signaling 3 (SOCS-3), and toll-like receptor-4 (TLR-4) expression (Figure 6A,B, $F(1,12)=7.67$, $p<0.05$, $F(1,12=25.9$, $p<0.001$, $F(1,10)=16.93$, $p<0.01$, respectively). There was also a significant main effect of exercise that increased expression levels of SOCS-3 and TLR-4 (Figure 6B, $F(1,12)=15.1$, $p<0.01$, $F(1,10)=26.12$, $p<0.001$) as well as a non significant trend effect on CCR2 and CXCL10 expression (Figure 5C, p=0.07, p=0.07). IL-1R expression was also increased but did not reach significance in the hypothalamus in the exercise + HF group (Figure 5C, p=.09).

Discussion

Exercise has been shown to have many beneficial effects on the brain and is a common treatment for obesity. The purpose of the first experiment was to demonstrate that short term exercise has the ability to alter reward related gene transcription. The purpose of the second experiment was to examine the interaction between high fat diet and exercise in feeding and reward regions of the brain. It was previously unknown if exercise was able to prevent neuroadaptations and behavioral changes that occur in obesity. Overconsumption of palatable foods not only drives obesity, but also has damaging effects on the brain. Chronic high fat diet causes circuitry changes in brain regions involved in food intake and decreases reward intake behaviors (Vucetic et al.,

2011a; Carlin et al., 2013). Exposure to a western-style diet is known to impair cognition (Beilharz et al., 2013) and cause neuro-inflammation that can impact food intake and learning and memory (De Souza et al., 2005, Jurdak et al., 2008). Our study demonstrates that exercise is able to regulate gene expression in these regions; however, it may not be an appropriate treatment for preventing reward dysfunction or neuroinflammation caused by long term high fat diet intake.

In the first experiment, mice were exposed to only two weeks of wheel exposure in adulthood. We found that with only two weeks of exercise, mice had decreased dopamine D1 receptor (DR1) and mu opioid receptor (MOR) expression levels in the prefrontal cortex. DA is hypothesized to be critically involved in movement and reward and increases in DA receptors in the striatum have been associated with increases in endurance capacity (Foley et al., 2006). Endogenous opioid levels have long been known to increase in the brainstem, caudate, and mesolimbic regions after exercise training (Boone et al., 1996; Werme et al., 2000, Sforzo et al., 1986, Janal et al., 1984). Mu opioid receptors mediate the increase in nociceptive thresholds and positive mood that occurs after exercise (Janal et al., 1984). However, our experiment shows the down regulation of both DR1 and MOR in the prefrontal cortex. This suggests differential regulation of DR1 and MOR in the prefrontal cortex compared to other brain regions. Difference in DR1 and MOR response to exercise may also be because of shorter exposure (2 weeks) to the running wheel and our use of mice instead of rats. Little was known about how exercise how exercise regulates gene expression at the molecular level and between in regions other than the hippocampus.

The likely mechanism behind down regulation of expression at this time point in the PFC is an increase DNA methylation at the promoter region of these genes. Global

DNA methylation was unaffected; therefore this suggests a specific signaling event and not a global down regulation of methylation levels. DNA methylation plays a crucial role in the remodeling of chromatin in the brain, and contributes to the regulation of brain plasticity and gene transcription. DNA methylation regulates expression in a highly context-dependent manner and a stimulus may have different effects at different genes (Suzuki et al., 2008). One possible mechanism by which exercise may be able to alter DNA methylation is through synchronous neuronal activation. Gou et al., (2011) demonstrated that electroconvulsive stimulation produced *de novo* methylation and DNA de-methylation throughout the genome; this could be similar to what is occurring after exercise. The up regulation seen after longer time points on exercise may reflect further remodeling events occurring after chronic exposure. Because exercise had the ability to affect expression of reward related genes, we next set up a longer experiment to look at exercise's interaction with chronic high fat diet.

For experiment 2, we were interested in the ability of exercise to prevent both behavioral and gene expression changes that accompany long term high fat diet. We gave mice access to the running wheel and to ad lib 40% fat diet concurrently. Our exercise + HF group gained less body weight than sedentary + HF animals, but were still significantly heavier than controls. Our lab and others have previously shown that intake of high fat diet causes decreases in dopamine and opioid related genes and this is associated with dysfunction in food intake and reward behaviors (Carlin et al., 2013; Vucetic et al., 2011, Johnson & Kenny, 2010). High fat/high sugar diets have been shown to alter behavior in rodents (Soulis et al., 2007, Prasad et al., 1996). For example, high fat/high sucrose diet decreased performance in learning and memory tasks (White et al., 2009; Stranaham et al., 2008) and decreased exploration of open arm in an elevated plus maze test (Anderson et al., 2013). In our model, mice did not show any

changes in anxiety behaviors after consumption of high fat diet and/or access to running wheel. This was interesting because in other models, such as mildly stressed rats, consumption of a high fat/high sucrose diet was anxiogenic (Legendre et al., 2006). Additionally, our study did not find any beneficial effects on anxiety-like behavior in exercised mice, while other studies have found exercise to be anxiolytic. For example, in humans, exercise reduces anxiety symptoms and is used as a treatment for both anxiety and depression (Herring et al., 2010; Dunn et al., 2005a), while exercise in rats can decrease anxiety in an open field test and elevated plus maze (Duman et al., 2008). However, the length of the exposure to the diet varies between labs and a 6 week exposure may not have been sufficient to elicit the anxiogenic response. Future experiments could address whether these animals response differently in a challenging or stressful situation to gain a more complete understanding of their stress reactivity.

Sucrose preference is a broad measure of natural reward intake and regulated by both dopamine and the endogenous opioid system. We found natural reward intake to be reduced in both exercise and high fat diet exposed animals. Sucrose preference can be diminished under a variety of different conditions, such as high fat diet intake (Carlin et al., 2013), chronic mild stress (Muscat et al., 1992), social deprivation (Berry et al., 2012) and lipopolysaccharide injection (Cross-Mellor et al., 2003). Sucrose preference is a model of anhedonia and diminished reward responsiveness in rodents. Potential mechanisms behind reduced sucrose preference after high fat diet could be reduced reward activation, reduced nutrient need, while reduced sucrose preference after exercise could be due to reward replacement. Both exercise and high fat diet are rewarding and we see access to one rewarding substance reducing of another rewarding substance, sucrose. Both high fat diet and exercise cause gene expression changes in reward related regions, therefore examining which genes parallel sucrose

preference will help us further understand the mechanism behind diminished reward intake in our model. Sucrose preference was diminished in animals given high fat diet, exercise, and exercise + high fat diet; therefore we can conclude exercise is not the optimal way to prevent the reward dysfunction that occurs in obesity. However, there does not seem to be an additive effect for HF and exercise for sucrose preference. Further studies are warranted to understand the mechanism driving decreased sucrose preference after two distinct and qualitatively different stimuli.

The mesolimbic dopaminergic pathway is activated by many rewarding behaviors, including the consumption of palatable foods and exercise. We examined gene expression of dopamine receptors in our experimental groups in reward related regions of the brain, nucleus accumbens (NAc) and prefrontal cortex (PFC). In the nucleus accumbens, both DR1 and DR2 expression were upregulated after 6 weeks high fat diet. Exercise alone did not alter the expression of dopamine receptors this region, even though we observed down regulation of DR1 after 2 weeks in the first experiment in the PFC. It may be the case a take a longer exposure is needed to alter receptors in nucleus accumbens. Furthermore, exercise was unable to normalize the expression of DR1 and DR2 in this region; and DR1 and DR2 remained elevated in exercise + HF animals. In the PFC of the second experiment animals, we saw a significant increase of DR2 expression after high fat diet and a trending increase in DR1. Exercise again was unable to normalize DR2 expression in the exercise + HF group. The PFC differs from the NAc in that there may be a possibility of an interaction for exercise and diet. Moreover, the pattern of expression does not parallel what we see in sucrose preference. Further examination of other neurotransmitter systems, such as the endogenous opioid system, involved in sucrose intake is necessary to see how exercise and high fat regulate natural reward intake behavior.

Epigenetic enzyme DNA methyltransferase 1 expression was also examined in reward related regions. We observed in experiment 1, that exercise may regulate gene expression by recruiting DNA methylation to specific promoter sites. Changes in promoter methylation have been observed in the brain, adipose tissue, and muscle after exercise training and may underlie the exercise mediated gene transcription involved in synaptic plasticity (Pareja-Galeano et al., 2014; Ronn et al., 2014, Gomez-Pinilla et al., 2011, Abel & Rissman, 2013). DNMT1 is highly expressed in the brain, catalyzes maintenance and de novo DNA methylation, and has been implicated in reward signaling (Gou et al., 2011, Day et al., 2013). In the NAc, there was a main effect of exercise on the upregulation on DNMT1. There was also an interaction between diet and exercise for DNMT1 in this region. High fat diet and exercise signaling both seem to converge to regulate DNMT1 transcription on their own. Interestingly, the combination of high fat diet and exercise normalizes DNMT expression. If DNMT1 is involved in the regulation of other genes involved in the damaging effects of high fat diet, exercise may have some benefit in normalizing high fat diet neuroadaptations. DNMT1 is not altered in the PFC in our second experiment model. Changes in DNMT1 activity levels may potentially be behind the changes in DNA methylation that occur after exercise in the short term. Further examination of this pathway will be needed to understand the activation pathway in both diet and exercise. At the transcriptional level at least, we see exercise is not able to prevent dopamine dysfunction and epigenetic regulation genes altered in obesity.

Obesity seems to have a negative impact on the brain and behavior. Obesity is associated with learning and memory deficits (Beilharz et al., 2013), dysfunction in reward behaviors (Johnson & Kenny, 2010), and diminished homeostatic control over food intake (Hellstrom et al., 2004). Obesity is also considered to be a chronically

inflamed state. One possible mechanism behind obesity's damaging effects on the brain could be neuro- inflammation in prefrontal cortex and hypothalamus. Some key markers of neuro inflammation is the increase in cytokine and inflammatory mediators that attract immune cells and activate microglia. C–C motif) ligand 2 (CCL2), chemokine receptor CCR2, and CXCL10 are part of the inflammatory chemokine response that elicit lymphocyte migration and trafficking into the brain (Prinz & Priller, 2010). Interleukin-6 (I-6) and IL-1β are increased in response to Toll-like receptor 4 (TLR-4) activation and are essential pieces of the inflammasome process (Manakan et al., 2012). SOCS-3 is a negative regulator of inflammatory cytokines as well as a negative regulator of the leptin receptor and could be the mediator leptin resistance found after high fat diet intake (Dunn et al., 2005b). Pro-inflammatory enzymes prostaglandin E synthase-1 (PGSE-1) and cyclooxygenase-2 (COX-2) synthesize neuro-inflammation mediators and perpetuate the inflammation cycle (Radler et al., 2013). These genes all together mediate the neuro-inflammatory response and the migration of immune cells into the brain, which may have damaging side effects. We demonstrated that inflammatory cytokines and inflammatory cytokine receptors are upregulated in the prefrontal cortex and hypothalamus after high fat intake and the effect of exercise may not be beneficial in all cases. In the PFC, inflammatory cytokines CCL2 and CXCL10 and cytokine receptor CCR2 mRNA was increased in animals fed high fat diet. Exercise alone had no effect on these genes; however, exercise in combination with high fat was able to normalize the inflammatory markers back to control levels in the PFC. We see a similar expression pattern occur with IL-6, PGSE1, which are increased after high fat diet exposure and normalized by exercise. Exercise has recently been shown to reduce neuroinflammation in the hippocampus (Kohman et al., 2013; Gomes de Silva et al., 2013). We have extended these finding to include the prefrontal cortex. Further experiments are needed

to see if this reverses and cognitive impairments associated with neuroinflammation in the PFC.

Exercise does not have a uniform anti-inflammatory effect in all brain regions, however. In the hypothalamus, we see that it is the combination of exercise and high fat to be the most detrimental. Although these results are preliminary because of small sample sizes in the high fat group, we see some informative patterns emerge. Exercise + HF animals had increased expression of cytokines CCL2, CCR2, CXCL10 as well as SOCS-3, TLR-4 and trending increase in IL-6. It seems that exercise actually exacerbates inflammation from high fat diet in the hypothalamus. Interestingly, SOCS-3 is upregulated by high fat diet and exercise as well as the combination of the two. Leptin resistance has been shown to be mediated by an increased expression of SOCS-3 and this could be associated with central inflammation in the hypothalamus and may mediate the observed dysregulated food intake and energy imbalance (Zhang et al., 2008; Cai & Lui, 2012; Milanski et al., 2004). In our study, 6 week of high fat alone was not sufficient to cause an upregulation of inflammatory markers other than SOCS-3. However, when coupled with exercise, high fat diet is able to elicit this response. Why the hypothalamus is so vulnerable to inflammation is unclear. It may be the region's highly specialized role in sensing energy utilization and metabolic activity that makes it particularly prone to possible oxidative stress and therefore, inflammation. The high level of vasculature and open blood brain barrier in certain hypothalamic areas may also play a role. This exacerbation of inflammation by exercise suggests that exercise may not be the optimal means to prevent the damaging central effects of high fat diet. This has implications for individuals who look to remain on a high fat diet while treating obesity with exercise. Ways to prevent neuroinflammation as well as dopamine dysfunction in this state will need to be further explored.

In summary, we demonstrated that a short exposure to exercise has the ability to alter dopaminergic and opioid gene expression and promoter methylation in a region not previously explored. This increase in promoter DNA methylation was specific to genes that were changed after 2 week running wheel exposure. Further, we have demonstrated that high fat diet causes decreases in natural reward intake and dopaminergic and inflammatory gene expression changes in the nucleus accumbens, prefrontal cortex, and hypothalamus. Behavior and gene expression were unable to be reversed by exercise and, in fact, exercise seem to have its own diminishing effect on sucrose intake. These gene expression changes lead us to believe that central reward signaling and epigenetic mechanisms may be altered after high fat diet intake and that concurrent exercise is unable to prevent these changes. We have also seen that the hypothalamus is particularly sensitive to the combination of exercise and high fat diet when it comes to inflammation. In conclusion, we believe that although exercise is extremely beneficial to cognitive enhancement and weight loss, other add on therapies may be needed to combat the neuroadaptations that occur in obesity and lead to reward dysregulation.

Figure Legends

Figure 1. Two weeks of voluntary wheel running decreased dopamine receptor D1 and mu opioid receptor gene expression and altered DNA methylation at promoter regions. A) Two week exposure to running wheel (black bars) decreased mRNA levels

of dopamine receptor D1 (DR1) and mu opioid receptor (MOR) compared to sedentary controls (white bars; n=6/group). Dopamine receptor D2 (DR2) was unaltered. B) Gene expression changes were associated with increases in DNA methylation at gene promoter regions of DR1 and MOR. C) Global DNA methylation in sedentary and exercised animals. Statistically different than controls at alpha level *p<0.05.

Figure 2. Animals with access to HF + exercise did not gain as much weight as sedentary animals on HF, but were still heavier than controls. A) Body weights at experimental end point were taken of sedentary controls (white bars), sedentary +HF (solid bar), exercise (vertical striped bar) and exercise +HF (checkered bar) groups (n=10/group). B) Body weight gain throughout 6 week was also analyzed. Statistically significant main effect of high fat diet at alpha level ****p<0.0001. Post hoc analysis between sedentary HF and exercise HF groups at significant level ##p<0.01.

Figure 3. Animals given access to running wheel and/or high fat diet did not show alterations in anxiety behavior. However, both exercised and/or high fat diet access reduced natural reward intake. Animals of sedentary controls (white bars), sedentary +HF (solid bar), exercise (vertical striped bar) and exercise +HF (checkered bar) groups (n=10/group) were tested in both the elevated plus maze (A), open field, (B) and sucrose preference (C) to assess anxiety behavior and natural reward intake. A) Time in open arm in elevated plus maze in seconds (s). B) Time in center arena of open field test in seconds (s). C) Percent preference of 4% sucrose solution to water. Main effect of high fat diet shown as significant at alpha level of,****p<0.0001. Main effect of exercise shown at alpha level of ^^p=0.01.

Figure 4. Gene expression of dopamine receptors and DNA methyltransferase are increased by high fat diet and not reversed by exercise in reward regions of the

brain. Gene expression was measured in the nucleus accumbens (NAc, A) and the prefrontal cortex (PFC, B) in sedentary controls (white bars), sedentary +HF (solid bars), exercise (vertical striped bars) and exercise +HF (checkered bars) groups (n=6-8/group). Fold change is calculated compared to sedentary controls. *p<.05 represents a significant main effect of diet, ##p<.01 main effect of exercise.

Figure 5. Gene expression of neuro-inflammatory related genes were increased by high fat diet and normalized by exercise in the prefrontal cortex. Gene expression was measured in the prefrontal cortex (PFC, A) in sedentary controls (white bars), sedentary +HF (solid bars), exercise (vertical striped bars) and exercise +HF (checkered bars) groups (n=6-8/group). Fold change is calculated compared to sedentary controls. A statistically significant main effect of high fat diet is shown at an alpha level *p<0.05, **p<0.01, ***p<0.001. Planned comparisons show significant difference between groups at an alpha level of #p<0.5, ##p<0.01, ###p<0.001.

Figure 6. Neuro-inflammatory related genes were increased by high fat diet and further increased by the combination of high fat diet and exercise in the hypothalamus. Gene expression was measured in the prefrontal cortex (PFC, A) in sedentary controls (white bars), sedentary +HF (solid bars), exercise (vertical striped bars) and exercise +HF (checkered bars) groups (n=6-8/group). Fold change is calculated compared to sedentary controls. A statistically significant main effect of high fat diet is shown at an alpha level *p<0.05. A statistically significant main effect of exercise is shown at an alpha level ^^p<0.01, ^^^p<.001. Planned comparisons show significant difference between groups at an alpha level of ##p<0.01

Table 1 Neuro-inflammatory gene expression in the prefrontal cortex. Summary of two-way ANOVA analysis of gene expression from the PFC preformed in sedentary,

high fat, exercise, and exercise + high fat groups. Interactions and main effects are presented alongside their p values.

Table 2 Neuro-inflammatory gene expression in the hypothalamus. Summary of two-way ANOVA analysis of gene expression from the hypothalamus preformed in sedentary, high fat, exercise, and exercise + high fat groups. Interactions and main effects are presented alongside their p values.

Figures and Tables

Figure 1.

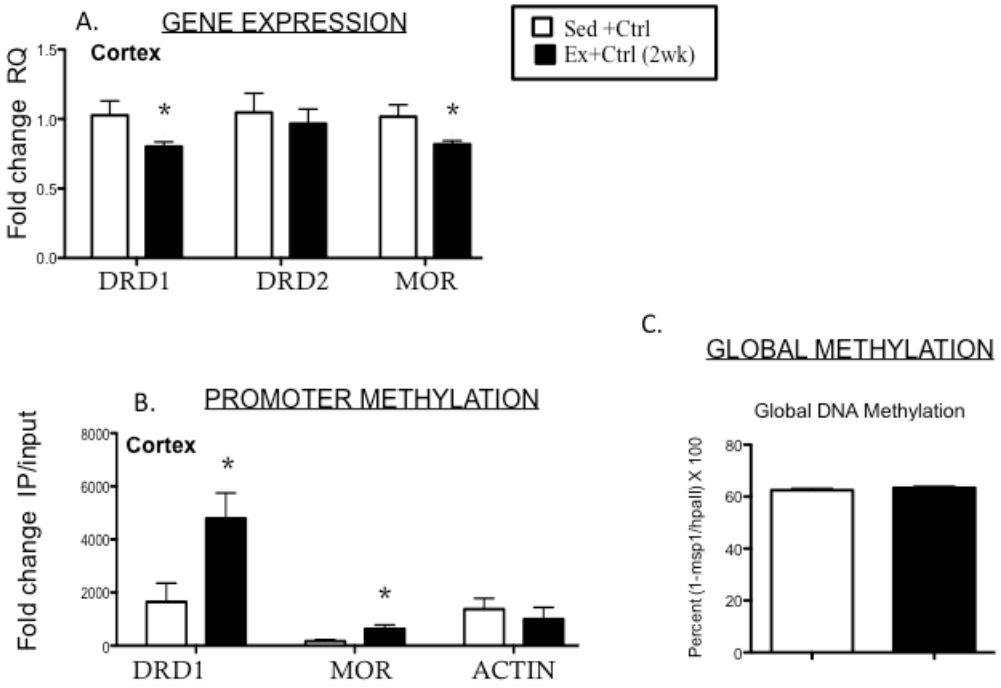
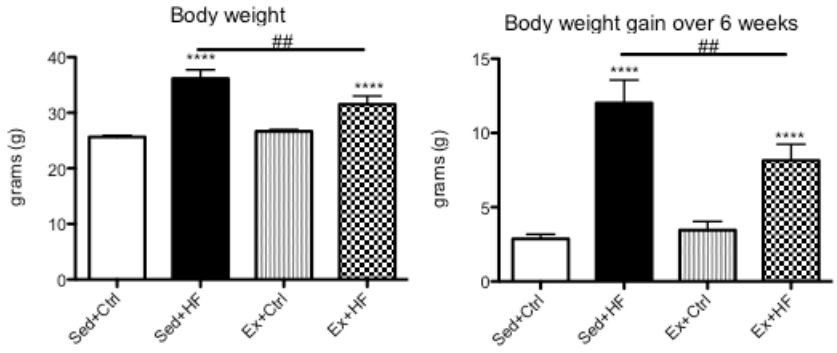


Figure 2

A.


Body weight
##
****
****
grams (g)
40
30
20
10
0
Sed+Ctrl
Sed+HF
Ex+Ctrl
Ex+HF
Body weight gain over 6 weeks
##
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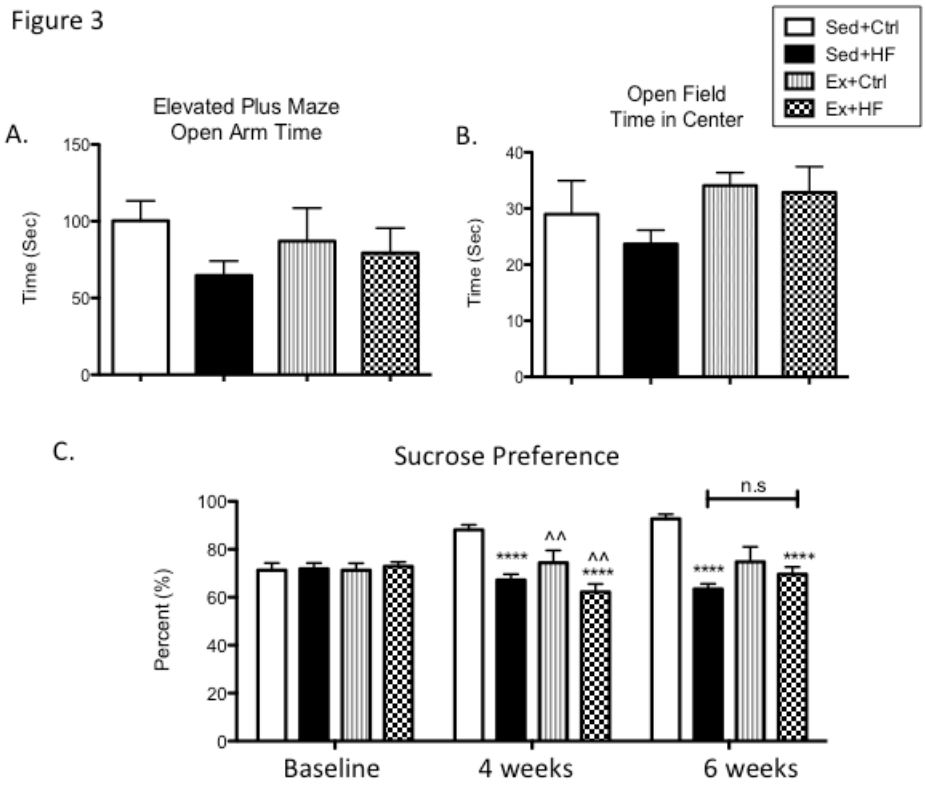
****
grams (g)
15
10
5
0
Sed+Ctrl
Sed+HF
Ex+Ctrl
Ex+HF

Figure 3


Sed+Ctrl
Sed+HF
Ex+Ctrl
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A.
Elevated Plus Maze
Open Arm Time
Time (Sec)
150
100
50
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B.
Open Field
Time in Center
Time (Sec)
40
30
20
10
0
C.
Sucrose Preference
Percent (%)
100
80
60
40
20
0
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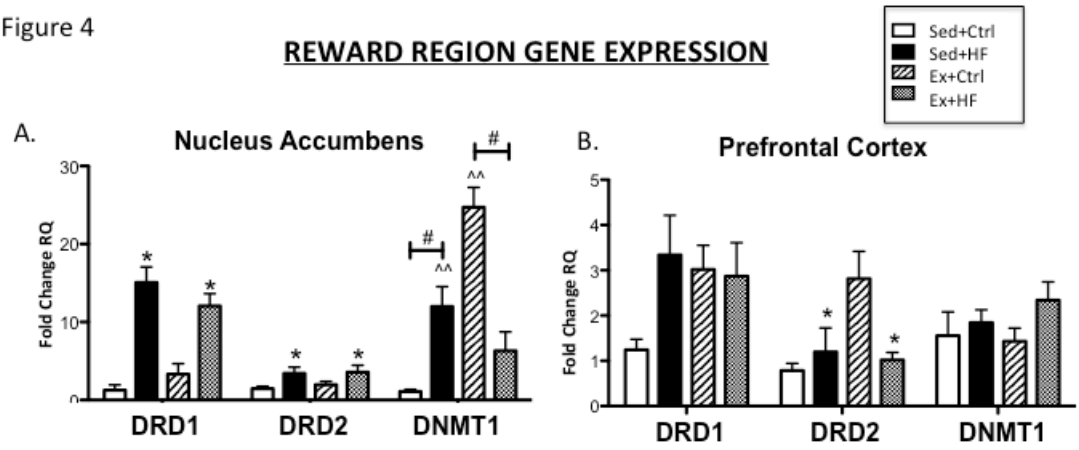
Baseline
4 weeks
6 weeks

Figure 4


REWARD REGION GENE EXPRESSION
Sed+Ctrl
Sed+HF
Ex+Ctrl
Ex+HF
A.
Nucleus Accumbens
Fold Change RQ
30
20
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DRD1
DRD2
DNMT1
B.
Prefrontal Cortex
Fold Change RQ
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DRD2
DNMT1

Figure 5 **NEURO-INFLAMMATORY RELATED GENE EXPRESSION**

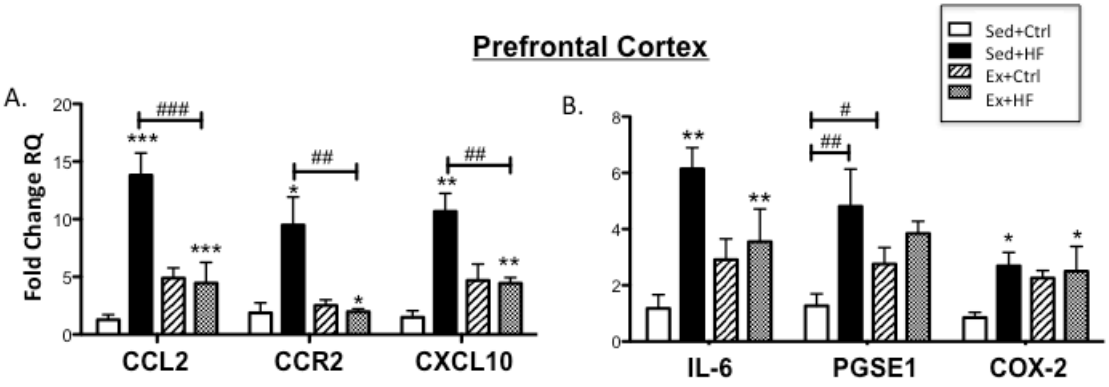

Figure 6 **NEURO-INFLAMMATORY RELATED GENE EXPRESSION**

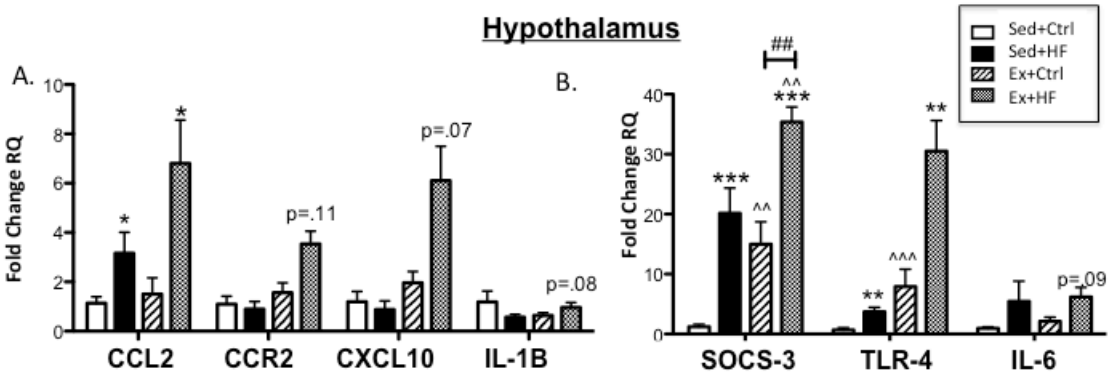

Table 1. Gene expression in PFC

Gene	Interaction	Diet effect	Exercise effect
CCL2	F(1,17)=20.4, p=0.0003	F(1,17)=17.7, p=0.0006	ns
CCR2	F(1,16)=6.58, p=0.021	F(1,16)=4.89, p<0.05	F(1,16)=4.63, p<0.05
CXCL10	F(1,17)=13.6, p=0.002	F(1,17)=12.15, p=0.0028	ns
IL-6	F(1,13)=7.00, p=0.02	F(1,13)=11.74, p<0.0045	ns
PGES	F(1,18)=15.73, p<0.0009	Trend, p=0.07	ns
COX- 2	ns	F(1,19)=4.88, p=0.04	ns

Table 2. Gene expression in hypothalamus

Gene	Interaction	Diet effect	Exercise effect
CCL2	ns	F(1,12)=7.67	ns
CCR2	ns	ns	Trend p=0.07
CXCL10	ns (p=.11)	ns	Trend p=0.07
SOCS-3	ns	F(1,12)=25.9, p=0.0003	F(1,12)=15.1, p=0.0022
TLR-4	F(1,10)=11.56, p=0.0068	F(1,10)=16.93, p=0.0021	F(1,10)=26.12, p=0.005
IL-6	ns	ns	ns
IL-1R	ns	ns	ns

CHAPTER 5: GENERAL DISCUSSION

Only 56 years ago, the prevalence of obesity was 13%. Today it is estimated that 69% of adults and 30% of adolescents are overweight (BMI 25-29.9) or obese (BMI > 30) in the United States (Ogden et al., 2011). The enormous toll that obesity takes in money and health from our society justifies an exploration of the drivers of the epidemic, the mechanisms of the disease, as well as the factors that aid in its prevention and cure. There are many drivers of the obesity epidemic, and it is now clear that this 5 fold increase over 50 years cannot be explained by genetics alone. Although certain genes can increase one's risk of obesity, an individual cannot become obese without the availability of calories to consume. The emergence of fast food establishments, a "snack food" industry and policies that favor corn and soybean production are just a few trends that have paralleled the obesity trend. Corn and soybean production is so inexpensive that an enormous supply of "junk" has been created using solely these two crops. Corn alone makes up over 25% of the food found on the supermarket shelves (Pollan, 2002). The inexpensive input has made it easy and profitable for companies to make food with high concentrations of sugar and fat that increase overconsumption. Companies that produce the combinations of highly concentrated sugar and fat have only been around 60 years or so. For instance, the first McDonalds opened in 1957, and the first marketed potato chip brand, Lays, began in 1938. The snack food market is a $46.1 billion industry and they produce highly concentrated sugar and fat products that are extremely difficult to resist. People are over consuming these modern manufactured foods and the consequences have been disastrous. Cheap, palatable foods are a permanent part of America's food landscape and its imperative for us find ways to lessen their impact on our health.

Why is it so difficult to say no to these manufactured, calorie-laden foods? We can imagine throughout the millions of years of human evolution, there was no need for the ability to suppress appetite. What was important was the ability to hunt and gather enough food to survive during lean times. We can imagine the brain to consider overeating of high-calorie food as beneficial, especially if you are uncertain of your next meal. Having large fat stores was a luxury, and the brain and body evolved ways to maintain them. Today, this behavior is no longer adaptive and is even counterproductive in a world where food is bountiful and energy-dense. The ability of these energy dense foods to override our homeostatic mechanisms leads to a brain-environment mismatch and increasing fat stores and declining health in many individuals. Obesity is associated with many chronic illnesses, such as cardiovascular disease, diabetes, osteoarthritis, and certain types of cancer. Fortunately, obesity is largely preventable and highly curable if we can only find ways to combat their effect on our brains. Even in the face of the facts and negative health outcomes, many people continue to over consume highly palatable foods, and thereby gain fat. Obesity, in this light, has often been compared to drug addiction. This comparison is highly controversial because of the negative associations accompanying the label “addiction” and many feel that one cannot be “addicted” to something necessary for survival. However, if the comparison is made on the neurobiological level and leads to better therapies and better drug targets, it may be a useful framework.

We have been introduced to the similarities between the neural circuits regulating feeding and drug addiction in chapter 1. Obesity neuroscience and drug addiction have previously been researched separately and addiction was identified as a disease much before obesity and manufactured foods were a problem. The inability to suppress intake despite negative consequences (e.g. foot shocks, cold environment) were shown with

cocaine intake studies first. Today, neuroscience is seeing the effects of highly palatable food mirror what was seen in the early addiction studies. Drugs of abuse hijack this extended motivation/feeding circuit by supra-natural activation and, over time, change it so other desires are dampened, and normal functioning is no longer possible. Obesity is also characterized by the unnatural hijacking of the dopamine motivation/feeding circuit. The foods we are eating today are unnaturally high in reward value and can stimulate and alter the brain in ways similar to drugs of abuse. Manufactured foods found in the modern diet contain concentrations of sugar and fat not found in nature and previously unavailable to human beings. Studying these calorie-dense foods like an addictive drug at the neurobiological level may be beneficial and lead to better obesity treatments.

Hypo-reward Hypothesis of Obesity

The hypo reward hypothesis of obesity is similar to the development of tolerance in addicted individuals. The hypo reward hypothesis postulates that there is diminished reward value for palatable foods in obese and obesity-prone individuals. The diminished reward value would necessitate more consumption in order to reach desired reward level. This is similar to tolerance that develops over time after chronic intake of drugs of abuse. An addicted individual needs to escalate his intake in order to achieve the desired effect. In chapter 2, 3, and 4 it was demonstrated that chronic high fat diet does indeed alter the mesolimbic dopamine region and reward behaviors. Four models of diet-induced obesity were examined in this dissertation. Chapters 2 and 3 examined different age groups on chronic high fat diet and used standard chow replacement as an obesity intervention in both male and female mice. We discovered that both age and sex were critical factors in the development and reversal of neuroadaptations seen in obesity. In

chapter 4, we examined exercise as an intervention for obesity and discovered how exercise can have its own effects on the brain that may not be entirely beneficial when paired with high fat diet. The decrease in dopaminergic gene expression, diminished dopamine neurotransmitter levels, and decreased sucrose preference were consistent with the hypo reward hypothesis of obesity and comparable to what is seen after long term drug intake. However, these changes did not necessarily show that chronic intake of high fat lead to further overconsumption. In fact, overconsumption of palatable food did not occur until acute or long term standard chow intervention and binge like consumption was only observed in females.

The hypo-reward hypothesis of obesity predicts that diminished inhibitory control paired with the decrease reward value of palatable foods will lead to further overconsumption of palatable foods. We saw decreases in dopaminergic genes and dopamine neurotransmitter levels in the prefrontal cortex as well as the nucleus accumbens. The prefrontal cortex is involved in inhibitory control of reward intake and dysfunction in this region is associated with impulsive behavior. Females were seen to have an imbalance of dopamine receptor DR1 and DR2 in the prefrontal cortex after standard chow replacement, which may be the mechanism seen behind the binge-like consumption of palatable food in this group. Our findings further strengthen the hypo-reward hypothesis of obesity. However, our results also highlight the importance of sex difference and nutritional status when examining palatable food consumption in the future.

Chronic high fat diet alters the dopamine reward system in an age and sex dependent manner.

Age Effects

We have investigated the age of onset and sex as critical variables in the neuro-adaptations seen after chronic high-fat diet intake. Taking the results of both chapters 2 and 3 we see that the timing of chronic high fat diet is a critical factor that affects the development of dopamine system neuroadaptations and adulthood reward behaviors. One behavior that was altered in an age dependent manner by chronic high fat diet was sucrose preference. Sucrose preference is a measure of natural reward and diminished sucrose intake is a mark of anhedonia in rodents and suggests decreased reward sensitivity. These results corroborate with other studies that show high fat diet is associated with depressive-like behaviors in rodents (Sharma et al., 2013). Males who began the high fat diet at birth and at pre-adolescence showed decreased sucrose preference; however, males who began the diet in adulthood had control level preference for sucrose. This suggests a critical time period from weaning (3 weeks of age) to pre-adulthood (before 6 weeks of age) to be extremely important for programming natural reward value in adulthood since the change was seen in both models of early hat fat exposure. High fat diet exposure during lactation has previously been shown to promote obesity in offspring (Tsuduki et al., 2013) and has implications for monitoring food consumption in lactating mothers in addition to monitoring food intake of offspring during early postnatal period. Interestingly, this age effect was not seen in females and all female groups had decreased sucrose preference. This suggests that all stages of female brain development may be vulnerable to high fat diet programming of reward dysfunction. This has implications for the treatment of obesity in the female and childhood obesity populations. If it is true that decreased reward value causes individuals who were obese as children and females to over consume palatable foods, interventions that can stop over consumption should be focused on these

populations. For example, strategies such as portion control at mealtime may be more beneficial than total caloric restriction or exercise in these groups.

We next explored how intake of another rewarding substance, high fat diet, and how it can regulate dopaminergic gene expression to elicit a decrease in reward sensitivity. Mesolimbic dopamine and opioid signaling after sucrose consumption determines the reward value of the sucrose solution. Dopamine signaling is mostly involved with the "wanting" aspect of sucrose intake, while opioid signaling mediates the "liking" aspect of sucrose intake (Mahler et al., 2011). In chapter 2 and 3 we see that dopaminergic genes were down regulated by chronic high fat diet in a sex and age dependent manner. However, because of the decrease in sucrose preference, we would also predict a down regulation of opioid signaling in the same regions. In fact, our lab has previously shown mu opioid receptor expression to be diminished after 20 weeks high fat diet in the VTA and NAc (Vucetic et al., 2011). The earlier high fat models also had more vulnerability in regards to changes in dopamine neurotransmitter levels. The difference in COMT regulation after high fat diet intake could be the basis behind the opposite regulation of dopamine (DA) neurotransmitter levels. It is also possible that the differences in COMT could be in response to the changes seen in total neurotransmitter levels. An earlier exposure to high fat diet also lead to decreased DA levels in VTA projecting regions NAc and PFC. High fat from pre-adolescence caused an increase in DA levels in these same regions. It seems that high fat diet from postnatal day 1 throughout lactation affects the expression of COMT, and therefore DA metabolism, in an opposing trajectory than pre-adolescence HF. Being able to observe dopamine circuitry at an earlier time point might give us insight to projections are developing at this time that can change the trajectory of how the brain responds to high fat diet.

Sex Differences

The ventral tegmental area was also a region where we not only saw an age effect, but also a significant sex difference. Dopamine transporter (DAT) was regulated by high fat diet in opposite directions in males and females. Chronic high fat diet caused decreased expression of DAT in males and increased expression of DAT in females in all of our DIO models. In chapter 2 we explored this sex difference further at the epigenetic level. Levels of DAT reflect the general state of dopaminergic function in the brain, and changes in DAT activity will alter dopamine function and dopamine dependent behaviors (Jaber et al., 1997; Amsterdam et al., 2007). A down regulation in DAT, as seen in DAT knockout mice, leads to an increase in dopamine levels and elevated motivation for sweet foods (Pencina et al., 2003). Although motivation for palatable foods was not assessed, elevated consumption of palatable food was seen in the DIO animals only after acute HFD removal. Palatable food consumption was mostly depressed by the presence of the high fat diet. However, females who received high fat earlier in life did not significantly decrease their palatable food intake in the presence of ad lib high fat diet. Additionally, pre-adolescent HF females reached binging levels of palatable food intake when HFD was removed. The sex difference seen in DAT expression in response to high fat diet may underlie the higher level of palatable food intake seen in pre-adolescent HF females. Indeed, it has been shown that female rats have a higher level of total DAT protein in the nucleus accumbens and striatum than males (Becker, 1999). It is also true that females are more sensitive to the rewarding effects of cocaine, a drug that binds to DAT (Zakharova et al., 2009). DAT is an important pharmacological target for obesity treatments as well as treatments for depression and attention-deficit hyperactivity disorder (ADHD) (Sakrikar et al., 2012; Ledford et al., 2010). Understanding the mechanisms behind how DAT expression is regulated in obesity will

be important when treating both male and female populations. It was not previously known that DAT was regulated by DNA methylation at the promoter region. Targeting epigenetic machinery may be a new drug target with the ability to reverse reward deficits seen in obesity. Insulin receptor/AKT/PI3kinase pathway also regulates DAT expression and dopamine homeostasis (Speed et al. 2011). This is also a potential target pathway where diet, hormones and metabolic sensing pathways intersect in the VTA and affect dopaminergic function. Future experiments will be needed to see if the insulin/PI3Kinase pathway is affected by high fat diet and converges on epigenetic proteins such as DNA methyltransferase-1(DNMT1) and alters transcription.

Dopamine Receptor Balance

Interesting gene expression patterns were observed in the nucleus accumbens and prefrontal cortex after high fat feeding. These areas are important for reward behaviors and impulsivity and are implicated in the control of feeding behavior as well as the administration of drugs of abuse. It has previously been shown by other labs that dopamine receptor levels are down regulated in obese models (Johnson & Kenny 2010, Ong et al., 2013, Alsio et al., 2010). We observed a down regulation in dopamine receptor D1 and D2 in the nucleus accumbens and prefrontal cortex in males and female mice on high fat diet in chapters 2 and 3. However we see a different pattern emerge after a shorter period of high fat intake in chapter 4. Mice in chapter 4 were placed on a 40% fat, 41% carbohydrate diet at 9 weeks of age for 6 weeks total. In that model we observed an increase in DR1 and DR2 in the nucleus accumbens and prefrontal cortex in this model. Differences in expression may possibly be due to starting the diet even later in life and for be maintained on diet for a shorter period of time. Additionally, a lower percentage of fat (40% vs 60% of calories) was used over the 6 weeks and this

exposure may not have been sufficient to down regulate dopamine receptors in these postsynaptic regions as we observed in chapters 2 and 3. It has previously been shown that 4 weeks of high fat feeding is insufficient to alter gene expression in the hypothalamus (De Leeuw van Weenen et al., 2009). Future experiments looking at earlier time points and different diet compositions will help us narrow down what molecules are responding to the high fat diet itself and which are homeostatic responses.

Dopamine impacts energy balance and reward behavior through activation of both DRD1 and DRD2 receptors. In the prefrontal cortex, this balance of DR1 and DR2 was disrupted in an age and sex dependent manner. It seems that the post-weaning to adolescence time period is critical in the development of normal dopaminergic circuitry in the prefrontal cortex. The prefrontal cortex forms later in development and early postnatal nutritional insults are poised to affect projections and proper synapse formation. Decreases in DR2 levels may have implications for increased risk for impulsivity and bingeing in obese individuals who were also obese as children. Drugs that block DRD2 enhance appetite and induce weight gain in animals and humans (Ader et al., 2005, Baptista et al., 2002). Lack of normal DR2 levels is associated with “reward deficiency syndrome”, which elicits exaggerated response to reward in the face of diminished signal transduction (Comings and Blum, 2000). Reward deficiency syndrome increases the risk of engaging in compulsive activities such as gambling, eating, and sex, and risk taking behaviors. These behaviors should be monitored in the obese populations and may be become an issue when trying to calorie restrict. Total dopamine levels (Giros et al., 1996), treatment by pharmacological agents (Buckland et al., 1992), as well as external rewards, such as sexual activity (Melis et al., 1995), and exercise (Foley et al., 2006) also all affect dopamine receptor expression level. We have since

added to these findings and show that chronic high fat diet alters dopamine receptor expression in a sex and time dependent manner.

Neurotransmitter Levels

Dopamine neurotransmitter levels were measured in nucleus accumbens nd prefrontal cortex after high fat diet intake. We observed the early life high fat diet to be important in altering neurotransmitter levels in adulthood; however diet exposure during the lactation period seems to be different than exposure during post weaning. High fat diet from birth leads to diminished DA levels in both males and females in adulthood, while high fat from weaning leads to increased DA levels in adulthood. Both age groups have diminished TH expression, however, opposite regulation of COMT expression in the VTA of animals that began the diet at birth and animals who began the diet at weaning. Looking at an earlier time point on the diet may give us clues to what is specifically altered in the lactation period that drives DA in one direction and what is affected in the post weaning period that drives DA in the other direction. We know that the sensitive window for DA regulation is closed by 6 weeks of age because Adult HF animal's DA levels remain unaffected by high fat diet.

Palatable food consumption was affected by presence of ad lib high fat diet.

In order to assess if high fat diet increased the consumption of palatable foods we gave mice access to high fat/high sugar food (peanut butter chips) for one hour every day and measured their consumption. We saw that consumption of the palatable food depended on the presence of ad lib high fat diet in both male and female animals. Animals decrease their total daily intake and their one hour palatable food intake when given high fat diet ad lib. Acute removal of the ad lib high fat diet causes normalization in

males and a binge-like consumption of palatable food consumption in females. Animals still had ad lib access to food and were not food restricted. Moreover, the acute removal of high fat diet during the experimental period is not a sufficient amount of time to reverse neuroadaptations seen in obesity. Therefore, we can see that the neuroadaptations caused by high fat diet increase the risk of over consumption during a diet withdrawal. Binge eating disorder is found in a subset of obese patients and is associated with dopamine dysfunction in the frontal-striatal (Davis & Carter, 2009). It is also thought that binge eating disorder arises in adolescence from development abnormalities (Michaelides et al., 2012). It is possible that high fat diet during this time can help program the propensity for bingeing later in life. Persistent changes occurring in the prefrontal cortex, either in dopamine neurotransmitter levels or decreased dopamine receptor levels could be the mechanism behind females reaching binge levels of consumption (>25% daily calories). These findings have implications for obese individuals trying to treat obesity with a low fat diet strategy and suggest that females may find this strategy more difficult. Which specific neuroadaptations are responsible for the increase in palatable food consumption are not yet known. These results highlight the need for targeted studies investigating the mechanisms of palatable food intake in both males and females.

Reversal of neuroadaptations by standard chow replacement depends on age of diet onset and sex

We demonstrated that it is possible to reverse the neuroadaptations and behavioral changes seen after high fat diet with a 4-week standard chow replacement. However, in some cases, we observed a pattern of persistence that depended on both age of diet onset and sex. A 4 week standard chow replacement was chosen as an our

first obesity intervention. Four weeks was a sufficient length of time to reverse behavioral adaptations but not a sufficient length of time to normalize body or fat pad weight. This allowed us to look at the effects of high fat diet itself while animals remained significantly heavier and did not go through the added stress of significant body weight loss. Sucrose preference was an example of behavioral normalization in diet induced obesity models before body weight returned to control levels. This suggests diminished sucrose preference was attributed to presence or intake of high fat diet itself and not increased body weight. The availability of high fat diet was also associated with diminished one hour palatable food intake. Once ad lib diet was removed, animals normalized or increased their one hour consumption of high fat/high sugar pellets. This down regulation was consistent with the hypo reward hypothesis of obesity that predicts a decreased reward value for food. However we do not see the risk of over consumption until removal of the high fat diet. Although we demonstrated the ability to reverse behavioral deficits, there were many instances where the neuroadaptations persisted after high fat removal. Moreover, we found that sex and age were critical factors that affected the persistence of these circuitry changes.

Dopaminergic Neurobiological Changes after Diet Removal

Many dopaminergic gene expression changes remained decreased after diet removal and were associated with the overconsumption of palatable foods. Gene expression after a 4 week standard chow replacement had an interesting pattern of persistence and reversal that varied by sex, brain region, and age of onset. Overall, the early high fat diet models seen in chapters 2 and 3 had a more persistent gene expression phenotype than the adult HF model. Females who began HFD from birth were less able to normalize gene expression changes in the VTA after standard chow

replacement. This inability to normalize after a 4 recovery period was also seen in dopamine receptors and DA neurotransmitter levels in the PFC of the males who began the high fat intake from time of birth. This implies the lactation period to be a particularly vulnerable period for male prefrontal cortex development and could have implications for cue induced feeding (Petrovich, 2013), self control (Alonso-Alonso et al., 2007), and food related decision making when "dieting" (Hare et al., 2009) in adulthood. One hour consumption of palatable food was not assessed in the high fat from birth group. Future studies will have to determine if high fat during lactation is a risk factor for over consumption during a dieting period.

In chapter 3, we further assessed the impact of age on the persistence of high fat diet brain and behavioral adaptations. We found that beginning high fat consumption earlier lead to more persistent changes after a standard chow replacement period. Pre-adolescent HF males had reduced dopaminergic gene expression in the VTA and NAc after standard chow replacement, while Adult HF males were able to normalize expression. Pre-adolescent HF males also had increased amount of DA and DA turnover in the NAc after standard chow replacement, whereas Adult HF males DA levels were unaffected. There was also a persistence of diminished gene expression in females who began the diet in pre-adolescence while adult HF females were able to normalize.

Dopamine Receptor Expression after Diet Removal

Removal of the high fat diet in females lead to a dysfunctional balance of DR1 and DR2 in the NAc in both pre-adolescent and adult exposed groups. In both the NAc and PFC, DR2 expression levels remained decreased while DR1 was able to normalize. This disruption of positive and negative control in dopaminergic tone was associated

with increase consumption of palatable foods. Females reached binge levels of consumption (<25% caloric intake) after a standard chow recovery period and this is possibly due to the increased risk of impulsivity that is associated with diminished DR2 tone (Johnson & Kenny, 2010; Noble, 2000). Withdrawing palatable food has previously been shown to heighten craving and motivation for sucrose and high-fat food as well as increase physiological and behavioral indices of anxiety (Sharma et al., 2013; Teegarden et al., 2007). High fat diet caused lasting neurochemical and behavioral changes related to dopaminergic function that have also been observed during chronic drug administration and their withdrawal (Russo et al., 2009; Robinson & Nestler, 2011). The diminished DR2 tone after HF withdrawal could have implications for altered consumption of drugs of abuse. For example, rodents on a high fat diet have exhibited reduced conditioned place preference for amphetamines (Davis et al., 2008), impairment of cocaine self administration (Wellman et al., 2007) and an obesity-prone rat strain has shown a reduced conditioned place preference for cocaine (Thanos et al., 2010). Disruption of the dopamine circuitry is implicated in the loss of control seen in both addiction and obesity (Volkow et al., 2011) and being able to reverse these neuroadaptations may be the key to obesity therapy.

Switching to a lower fat or lower calorie diet is the first line of defense against obesity; however, long term compliance on a new diet is the reason behind high failure rates of “diets”. It is possible that the neuroadaptations that persist after long term high fat consumption contribute to dietary relapse. Understanding how age and sex impact brain and behavior in the high fat withdrawal stage will have implication for obesity management and interventions. We know now that the obese brain is markedly different than the lean brain and these changes can predispose one to overconsumption once

high fat diet is removed. Since both pharmacological and behavioral therapies are often combined with diet replacement, understanding the brain during this switch and factors that can raise adherence rates will help the success of therapies in the future.

Exercise does not reverse reward dysfunction and neuroinflammation seen in obesity

Our experiments in chapter 2 and 3 compared the physiologic, behavioral, biochemical, and molecular alterations occurring after high fat diet intake and acute and long term withdrawal in both males and females at different ages of exposure. We demonstrated it is possible to reverse some of the neuroadaptations and reward dysfunction that occurs in obesity by replacing HFD with standard chow. However, there were some instances were adaptations persisted and this suggests that some populations may be more resistant to central normalization with dieting. Chapter 4 explores exercise as an additional way to prevent the neuroadaptations caused by high fat diet. Exercise has the ability to activate reward regions of the brain and alter reward behavior (Bothe et al., 2013; Greenwood et al., 2011). Many of the studies demonstrating the cognitive benefits seen after exercise were focused on the learning and memory in the hippocampus. Exercise is now a potential treatment option for addiction disorders (Smith & Lynch, 2011). However, it was previously unknown if exercise could prevent the dysfunction seen in reward related regions in obesity. We found that although exercise has the ability to reduce body weight in obese individuals, it was not an optimal treatment for reward dysfunction in obesity. Exercise was unable to prevent the increases in dopamine receptor expression and decreases in natural reward consumption caused by high fat diet. This has implications for obesity treatment regimes. Exercise alone would not be sufficient to treat reward dysfunction in obesity

and additional therapies should be added. Our study examined exercise in conjunction with a high fat diet; it is possible that a low fat diet is needed with exercise to prevent reward dysfunction after high fat diet intake.

We also examined the ability of exercise to inhibit neuroinflammation seen in the brain after high fat diet intake. Obesity is increasingly being linked with a sub-clinical inflammatory state, as seen by higher than normal levels of pro-inflammatory mediators such as the cytokines interleukin (IL)-1β and tumor necrosis factor (TNF)-α in the circulation of chronically obese individuals (Somm et al., 2006, Mantzoros et al., 1997). It is now known that this is due in part to expanding adipose tissue, major source of cytokines and cytokine-like hormones, such as leptin (Trayhurn & Wood, 2005). Expanding adipose tissue and the high fat diet itself can both increase the production of inflammatory markers. Although the cellular and molecular pathway underlying how the high fat diet increases cytokine production are unclear, studies have demonstrated that saturated fatty acids can directly stimulate TLR-4 (Milanski et al., 2009). If direct stimulation of the inflammatory response is possible by fat molecules from the diet, an intervention of exercise and a low fat diet, not exercise plus a continuation of the high fat diet, should be the recommended way to combat neuro inflammation in obesity.

We observed that intake of high fat diet caused an increase in inflammatory markers in the hypothalamus and prefrontal cortex. Inflammation in these regions could disrupt feeding and reward behaviors and maybe even lead to problems with impulse control. Exercise has many beneficial effects on the brain and is a first line of treatment for obesity. Exercise has the ability to normalize expression of inflammatory markers in the prefrontal cortex, but may have an additive effect in the hypothalamus. Exercise has beneficial effects on adiposity in the periphery, but would not be able to decrease the damaging effects of central inflammation. Exercise's inability to normalize reward

dysfunction and neuroinflammation has implications for future obesity treatments. It may be recommended that individuals switch to a lower fat diet while exercising or use supplementation with anti-inflammatory medications during intervention. We observed an interaction with exercise and high fat in some brain regions. However, studies show that the quality of fat in the diet is just as important as the quantity. The ratio of arachidonic acid (AA) to docosahexaenoic acid (DHA) is important for pro-and anti-inflammatory processes, and supplementation with DHA may be beneficial for the treatment of neuroinflammation (Bazan, 2005; Serhan, 2005). The experimental diet was high in saturated fat to match the western diet eaten by people today. It is now known that diets rich in saturated fat increase oxidative stress in the brain (Stranaham et al., 2011) reduce neurogenesis (Lindqvist et al., 2006), and enhance neuroinflammation (Thaler et al., 2012). It could be recommended in the future that if individuals are to remain on high fat diet while exercising, DHA or fish oil supplement is necessary to prevent the exacerbated inflammatory response seen in certain brain regions.

High fat diet and exercise cause lasting changes in gene expression possibly through the recruitment of epigenetic machinery.

Results from these experiments demonstrate that early life nutrition can have long-lasting genetic and behavioral consequences that are linked to alterations of the epigenetic state. Our results suggest that there may be multiple sensitive windows throughout development that can change the trajectory of reward circuitry development. One way a sensitive window could occur is through chromatin remodeling over development leaving some genes open for both transcription and modification of transcription levels. Some genes may no longer be vulnerable as the brain develops and

some might continue be vulnerable way into adulthood. Another important factor determining sensitive windows would be the timing of development of each specific brain regions. The PFC continues to develop throughout adolescence and maybe open to remodeling later in life, whereas a more caudal region like the VTA is fully developed. For instance, we observed high fat from birth lead to increased DNA methylation at the DAT promoter region in the VTA. On the other hand, a different stimulus of short term exercise beginning at 9 weeks of age increased DNA methylation at DR1 and MOR promoter in the cortex. We also observed more persistent dopaminergic gene expression changes in the prefrontal cortex of early high fat from birth than in the PFC of animals receiving high fat from adulthood. Each brain region has different levels of plasticity and sensitive time periods that can alter the developmental trajectory of neurotransmitter system will vary over space and time.

Epigenetics is viewed as the way through which the environment interacts with an individual's genome to determine aspects of gene expression. A subset of epigenetic changes is very stable, which makes them ideal mediators both of reward system vulnerability and of the long lasting drug or food-induced brain maladaptations that underlie an addiction disease. DNA methylation was classically thought of as a very stable means of gene repression (Klose & Bird, 2006). However we show that DNA methylation at the DAT promoter region was reversible with 4 week standard chow replacement in males starting high fat from birth. The de-methylation step may be an additional hurdle to overcome before gene expression can return to normal levels. This time needed to demethylate the promoter region could account for the persistence of gene expression changes seen in pre-adolescent high fat animals after standard chow replacement. How the high fat diet and exercise interact with DNA methyltransferase on a molecular level is currently unknown. However, identifying

sensitive windows for epigenetic modification can point to time periods where drugs acting on these pathways may be more beneficial and prevent chronic illnesses, like obesity, in the future.

We demonstrated that exercise was unable to reverse the dysfunction in natural reward intake seen after high fat diet consumption. In fact, exercise alone reduced sucrose preference similar to high fat diet. Since exercise and high fat are both rewarding and increase dopamine levels in the mesolimbic region of the brain, it is possible they elicit neuroadaptations through a similar pathway. One common mechanism that exercise and high fat diet may converge on is regulation of DNA methylation at promoter regions to elicit gene expression changes. Exercise and high fat intake do not alter global DNA methylation, however global methylation assays detect intergenic and genome wide levels of methylation. Exercise and high fat seem to work to alter DNA methylation at the promoter region to regulate gene expression in the specific genes we examined. It is possible that other genes promoters were altered by DNA methylation in a similar manner even though global methylation levels remained unchanged.

DNA methyltransferase 1 (DNMT1) is involved mainly in maintaining existing DNA methylation patterns and its activity is regulated by phosphorylation. The N-terminal region of Dnmt1 is known to interact with various proteins, such as methyl-CpG-binding protein 2 (MeCP2),and various protein kinases (Kamesita et al., 2008). Further molecular studies are needed to analyze the specific pathways involved in both high fat and exercise that converge on DNMT1 to regulate gene expression. One possible upstream pathway previously shown to be involved in both reward mechanisms and epigenetic modulation would be the PI3Kinase/AKT phosphorylation cascade that can activate DNMT1 (Speed et al., 2011; Mei et al., 2010; Day et al., 2013). Understanding

this pathway could give us new potential targets for the treatment of obesity and its associated reward dysfunction. Our results on both diet withdrawal and exercise suggest new directions in the development of more successful therapies for obesity prevention. We demonstrated the importance of finding additional obesity interventions and further appreciate the potent rewarding effects of both palatable food and exercise on the brain.

Limitations

Other Neurotransmitter Systems

As we attempted to answer how age and sex affected the response to high fat diet and the response to obesity intervention such as high fat withdrawal and exercise, many more questions were created in the process. The hypo reward hypothesis of obesity predicted the diminished sucrose preference we observed after high fat diet, however we did not find a good candidate gene that predicted diminished sucrose followed by reversal after a standard chow replacement. This could be because the rewarding aspects of sucrose come from both dopamine and the endogenous opioid system. Our lab has previously shown diminished MOR levels after high fat diet that was associated with increases in DNA methylation at the promoter region (Vucetic et al., 2012). However, it is currently unknown how MOR may respond to a standard chow replacement. Levels of MOR in the withdrawal period would give us a better understanding of both sucrose preference and one hour palatable food intake. Moreover, sucrose preference and palatable food consumption do not give a full picture of the "wanting" aspect of reward. Experiments such as progressive ratio training can be compared to dopaminergic gene expression and help us better understand the motivational state of being on and off the high fat diet.

Functionality of Gene Expression Changes

Examining the full functionality of gene expression changes was also a limitation of this set of experiments. Dopamine receptors were altered in response to high fat diet; however, we did not see any alterations in dopamine receptor signaling. There was no difference in response to a DR1 agonist locomotion or c-FOS induction (data not shown). However, a dose response to DR1 agonist was not performed and it is possible we may see differences in sensitivity to DR1 agonist at additional doses in future experiments. Additionally, it is known that dopamine receptor mRNA levels can change in response to increasing or decreasing dopamine neurotransmitter levels in the examined regions. Downstream signaling at the c-FOS induction step could have been up regulated to account for the decrease in receptor levels and the response remained at normal levels. There were no functional experiments performed for DR2. DR2 signaling is extremely important for over consumption behaviors and analyzing DR2 signaling in addition to mRNA levels would have given us a richer picture of the reward system on high fat diet.

Sex Hormones

An additional limitation to this study is the unknown level of sex hormones in animals at time of diet onset. We can assume that animals beginning high fat diet at birth had lower levels of both estrogen and testosterone than animals in adulthood. The differences we see between age of onset and sex could be due to a complex interaction with levels of sex hormones changing throughout life. All animals were adults during data collection and measurements occurred at the same time each day to minimize variance that occurs across the circadian cycle. One future way to incorporate hormone levels into the analysis would be to measure both estrogen and testosterone at start of diet onset and see if hormone level correlates with body weight changes and gene

expression changes in adulthood. One way to control for variations in hormones over time would be to monitor female cycling and only take measurements of females in the same point of the estrus cycle. Since we know estrus cycle is important for administration of drugs of abuse, like cocaine (Roberts et al., 1989) it may also be important for the intake of sucrose or palatable foods. We saw that both early life and adult exposed females had reduced sucrose preferences and increased levels of DAT expression after high fat diet intake. Seeing if these sex differences remain at all time points of the estrus cycle or in ovarectomized animals will give us insight into the importance of sex hormones in the central response to high fat diet.

Obesity Resistant Animals

Another limitation to this study was not observing metabolic and behavioral effects before onset of diet. When mice are placed on a high fat diet, often times there are some obesity resistant animals as well as highly obesity prone animals in the group. Examining the behavioral correlates in the two extremes (obesity resistant and obesity prone before and after chronic intake would have been another interesting and informative way to analyze the data. We did not find a high level of obesity resistant mice in our experimental model in order to power the proper analysis. This could have been due to the high level (60%) of dietary fat eaten, which is known to quickly drive obesity in our mouse strain. Obesity resistant animals may be more prevalent at a lower level of dietary fat obesity model. Even without this analysis, our results provide possible risk factors that may predict adherence to dietary intervention.

Analyzing the time point before diet intake would have provided us with additional risk factors that predict extreme obesity and hyperphagia or factors that predict obesity resistance at different times of development. Looking at metabolic and adiposity data at

earlier time points on the diet could have provided us with further understanding on adiposity development in younger aged mice. Both brain development and fat cell development are occurring at the same time. It is possible that early life adipose tissue is just as vulnerable to the nutritional environment as the brain. If beginning high fat diet earlier in life leads to more or less fat cells that could mean possible differences in leptin signaling to the reward regions and therefore an altered dopaminergic tone.

Lactation Period

Chapter 2 examines the response to high fat diet and withdrawal in animals exposed since birth. One confounding factor of this study is availability of the high fat diet during the lactation period. This would mean the mother was also exposed and the offspring were receiving the high fat diet through the mother's milk. It is difficult to compare the diet induced obese animals in chapter 2 with the other experimental models in chapters 3 and 4. The data are still extremely valuable because of how important the lactation period is for the developing brain. However, it is difficult to tease apart the maternal diet and the effects of the high fat diet in offspring when looking at data later in adulthood. We controlled for this confounding factor in later experiments and compared high fat beginning from weaning and high fat beginning at six weeks of age.

Age of Exercise Exposure

In chapter 4 we examined both a short term and long term exposure to exercise and its effect on behavior, dopamine receptor levels, and neuro inflammatory markers. We also examined the interaction of long term exercise with long term high fat diet. One limitation to this study is not being able to compare the high fat diet effects in chapter 4 with the high fat diet effects observed in chapters 2 and 3. Animals in the exercise and

high fat diet study received different diet composition, had a shorter length of time on the diet, and began intake at a much later age (9 weeks). The differences in results from our exercise model compared to our other DIO models highlights how important it is to control for all these variables in future studies.

High Fat Diet Composition

The composition of the diet is another limitation of these experiments. Animals received ad lib exposure to 60% or 40% high fat diet. Although these diets quickly and reliably drive obesity, these formulations are not an exact model of the modern diet. The typical American diet is 50% carbohydrate, 15% protein, and 35% fat (Last et al., 2006) and people are provided with a wide variety of choices. There is also the enormous presence of food cues to consider, such as advertisements and cultural norms weaved throughout the day that can prime the individual and increase the consumption of food. It has been demonstrated that humans habituate when given the same food items and decrease intake when presented with them daily. But when presented a variety of food choices, people tend to increase their intake (Epstein et al., 2011). We observed a similar phenomenon in our animal models. Animals on the ad lib 60% high fat diet have decreased daily intake of their ad lib chow (data not shown). However, when the diet is removed, and they are presented with a new palatable food choice, they rapidly normalize or increase their intake. This decrease of high fat diet intake after 12 weeks means that animals may be habituated or "bored" with the diet. The habituation to the diet could be a factor of the hypo-reward state of obesity or a parallel characteristic. Other labs have used a "cafeteria" diet that may mirror the variability seen in modern human diet better than the homogenous 60% diet used in our study. If further studies show that a variety of tastes increase energy intake and drive obesity, this could have implications for school menu planners, public health officials, a nutritionist that find

combating obesity important. Suggesting that people stick to a routine and the same foods may be a good strategy, in conjunction with caloric restriction and exercise, for obesity treatment.

Interaction between Voluntary Exercise and High Fat diet

No previous study has examined the interaction of high fat diet and exercise in reward regions of the brain or the interaction with regard to neuro inflammation. Since both palatable food consumption and physical activity have actions at the mesolimbic dopaminergic system, our attention focused on the influence of chronic exercise in the prevention of the neuroadaptations seen in obesity. However, we found that exercise had its own effects on reward behaviors and the dopaminergic gene expression. Furthermore, we did not find any strong effect of exercise or high fat diet on anxiety behavior; although this has been shown in other models. This could be due to differences in rodent species, housing conditions (singly versus communal), duration of exercise, and exercise intensity. One big limitation of this study was lack of information on the length and distance the animals ran. Animals were given voluntary access to the running wheel and there is a chance that some animals did not obtain sufficient amount of exercise per day in order to see behavioral changes after 6 weeks of exposure. Although a large amount of exercise is not necessary to see behavioral effects. In fact, previous studies have shown that a minimum of 8 wheel revolutions per day was enough to increase leptin sensitivity and decrease body weight on a control diet (Shapiro et al., 2011). One way to control for variation between running animals would be to use forced running instead of voluntary running so all the animals have equivalent speed, frequency, duration, and intensity of exercise exposure. Forced exercise is often thought to be an added stressor to the animal. However, other researchers have suggested that forced exercise more closely models the average human exercise

regimen, while a voluntary exercise may only model highly motivated endurance athletes (Leasure et al., 2008). Data on intensity of exercise and distance travelled could help stratify the analysis into high and low volume runners and we would possibly see predicted anti-anxiety effects.

Sex Differences in Exercise Response

Another limitation to the exercise study is that it was only performed in males. We have seen from chapters 2 and 3 that there are many sex differences in response to high fat diet and would predict sex specific responses for exercise exposure. Other labs have shown sex specific responses to exercises, such as the reduction of cocaine administration after exercise in females (Cosgrove et al., 2002). Voluntary wheel running has also been shown to increase extinction and attenuate reinstatement of cocaine self-administration in adult female rats (Zlebnik et al., 2010). These results suggest that females may benefit more from exercise in abolishing reward dysfunction after high fat diet intake. Further studies in female mice are needed to confirm this prediction.

Additionally, it is possible that exercise may have a more beneficial effect in prevention of reward dysfunction before high fat diet onset rather than concurrently with high fat diet intake. One important experiment would be to see if exercise exposure in adolescence prevents future reward dysfunction when placed high fat diet in adulthood.

Implications and Conclusions

As the number obese individuals continue to rise every year, many people are seeking answers for why so many people struggle with overconsumption and why there

are such high failure rates for weight loss. Although obesity is the consequence of imbalanced caloric intake and energy expenditure, diet and exercise alone have not been enough to combat most cases of obesity. Decreased caloric consumption and increased physical activity can be effective in normalizing weight in the short term; however these lifestyle modifications have proven very difficult to sustain (Volkow et al., 2007). When attempting to diet, obese individuals initially lose weight; however, this weightloss slows down until about an average of six months where then weight begins to return back to baseline (Jeffery et al., 2000). Adherence to the weight loss strategy is the most difficult part to curing obesity and individuals often "relapse" back to old, unhealthy eating habits (Knowler et al., 2009). Current pharmacologic treatments for obesity have failed to adequately address the problem and seem to only have short term benefits (Yanovski, 2005). Our experiments suggest that the obese brain is markedly different with than the lean brain and changes along with BMI changes. Drugs that work in the short term may no longer be effective after significant weight loss. We also demonstrated that sex differences and age of obesity onset are other potential factors behind the failure rates of pharmaceutical interventions. Characterizing how the dopamine system responds to the development of obesity and to obesity interventions is important for understanding what factors lead to increased success rates in weight loss. The development of effective weight loss interventions requires a thorough understanding of the factors that drive and/or prevent the overconsumption of palatable food. Obesity is increasingly recognized as a behavioral disorder and certain populations, such as females and adults who were obese as children, may benefit from adjunct behavioral strategies to combat over consumption.

We demonstrated that early life periods such as lactation and pre-adolescence periods in the mouse are important factors in the response to high fat diet and

intervention. High fat diet caused more neurochemical and gene expression changes in the dopamine system if started earlier in life. These changes tended to be more persistent and may put adults who were obese as children at increased risk for failure when attempting to diet. The lactation period in the mouse is not equivalent to the lactation period in humans because of the timing of brain development. The lactation period in mice is more equivalent to the third trimester in humans. This has potential implications for pregnant mothers to maintain healthy eating habits throughout pregnancy and for parents and schools to help instill healthy eating habits in their children.

As adults, the most important issue is maintaining the weight reduction. Our results have implications for adults who were obese as children and are beyond the preventative stage. It might be beneficial for this population to know that reversal of central dysfunction might take longer than the rest of the population, and additional strategies may be required when trying to lose weight. For instance, psychotherapy could be useful to help combat dysfunctional reward behaviors in combination with diet and exercise. The view that obesity is caused by a lack of willpower or poor self-control is extremely prevalent, and promotes the stigmatization of overweight and obese individuals. These results demonstrate that the high fat diet itself is acting on the brain to change it and promote overconsumption. High fat diet can activate epigenetic machinery to make these changes last very long and be more difficult to reverse. In addition to the scientific value of these studies, further promoting that obesity is a disease caused by environmental factors similar to addiction, may help the psychology of obesity patients and direct attention away from the poor “character” or “morality” stereotypes that are associated with the obese state.

Our results demonstrate that high fat diet has the ability to cause neuroadaptations in the reward pathway such as changes in gene expression and dopamine neurotransmitter levels. Altering levels of dopamine and dopamine signaling may have implications in drugs acting in on these proteins. It is possible that if tested under the right conditions, we would see an altered response to drugs such as cocaine and amphetamines. We would predict a decrease in the rewarding effect of cocaine in males and increase in the rewarding effect of cocaine in females. This can be tested through cocaine place preference or progressive ratio tests for cocaine administration. Furthermore, increases or decreases in dopamine transporter levels seen after high fat diet would also have implications for drugs already on the market that target this protein, such as methylphenidate and bupropion, used in the treatment of Attention Deficit Hyperactivity Disorder (ADHD) and depression. This may have implications for treating obese patients and they may need different dosing schedules that depend on sex and age of obesity onset. The link between obesity and depression has been repeatedly established (Luppino et al., 2010). We see that high fat diet affects the pathway implicated in both addiction and major depression. Researchers looking to target dopaminergic proteins in these regions for obesity treatment will have to be cautious of the potential addictive or depressive effects that can occur. In fact, in the case of Rimonabant, this anti-obesity drug failed to make it through clinical trials because of the increase in suicide rates that were associated with its usage.

Obesity is a complicated state that is associated with increases in adiposity, changes in hormone levels, inflammation, and neuroadaptations that affect the homeostatic and hedonic food intake systems. We see from our study that just one intervention may not be sufficient to prevent all these symptoms. Although both exercise and dieting have beneficial weight loss effects, they may not be optimal treatment on

their own in curing the reward dysfunction and neuro-inflammation occurring after high fat diet. Combinations of treatments together or at different time points may be the most effective way to treat obesity. Additionally, as BMI decreases due to intervention, other adjunct therapies may be necessary. Experiments studying how the brain changes in response to high fat diet and weight loss will help us work out the optimal age and sex specific targets for treatment.

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